Coenzyme q biosynthesis: coq6 is required for the c5-hydroxylation reaction and substrate analogs rescue coq6 deficiency.

Mohammad Ozeir, Ulrich Mühlenhoff, Holger Webert, Roland Lill, Marc Fontecave, Fabien Pierrel

Elsevier Editorial System(tm) for Chemistry & Biology
Manuscript Draft

Manuscript Number: CHEMISTRY-BIOLOGY-D-11-00115R1

Title: Coenzyme Q biosynthesis: Coq6 is required for the C5-hydroxylation reaction and substrate analogues rescue Coq6 deficiency

Article Type: Research Paper

Keywords: coenzyme Q; ubiquinone; monooxygenase; ferredoxin; Q deficiency; 4-hydroxybenzoic acid; analogues; Coq6; hydroxylation; Q biosynthesis; Coq8

Corresponding Author: Dr FABIEN PIERREL, Ph.D.

Corresponding Author's Institution: CEA-CNRS-UJF

First Author: Mohammad Ozeir

Order of Authors: Mohammad Ozeir; Ulrich Mühlenhoff, Ph.D.; Holger Webert; Roland Lill, Ph.D.; Marc Fontecave, Ph.D.; FABIEN PIERREL, Ph.D.

Abstract: Coenzyme Q (Q), an essential component of eukaryotic cells, is synthesized by several enzymes from the precursor 4-hydroxybenzoic acid. Mutations in six of the Q biosynthesis genes cause diseases that can sometimes be ameliorated by oral Q supplementation. We establish here that Coq6, a predicted flavin-dependent monooxygenase, is involved exclusively in the C5-hydroxylation reaction. In an unusual way, the ferredoxin Yah1 and the ferredoxin reductase Arh1 may be the in vivo source of electrons for Coq6. We also show that hydroxylated analogues of 4-hydroxybenzoic acid, such as vanillic acid or 3,4-dihydroxybenzoic acid, restore Q biosynthesis and respiration in a Saccharomyces cerevisiae coq6 mutant. Our results demonstrate that appropriate analogues of 4-hydroxybenzoic acid can bypass a deficient Q biosynthetic enzyme and might be considered for the treatment of some primary Q deficiencies.

Suggested Reviewers: Catherine F Clarke Ph.D.
Professor, Department of Chemistry and Biochemistry, University of California Los Angeles
cathy@chem.ucla.edu
Prof. Clarke has discovered the function of most of the genes involved in coenzyme Q biosynthesis in yeast. She then carried out in vitro work with some Coq proteins. She is therefore highly competent to evaluate the results presented in our paper.

Agnès Rötig Ph.D.
INSERM researcher, Hôpital Necker-Enfants Malades, Université René Descartes Paris V
agnes.rotig@inserm.fr
Dr Rötig has identified mutations in several genes of coenzyme Q biosynthesis which cause primary CoQ deficiencies in humans. Besides her profound knowledge of CoQ biosynthesis, Dr Rötig will be in a position to evaluate the relevance of our results for potential application to human CoQ deficiencies.

Pavel J Sindelar Ph.D.
Department of Molecular Medicine, Karolinska Institutet
pavel.j.sindelar@gmail.com
Dr. Sindelar published last year a paper in Nat. Chem. Biol. in which he describes an inhibitor of

coenzyme Q biosynthesis in mammalian cells. This inhibitor, 4-nitrobenzoic acid (4-NB)is an analogue of 4-hydroxybenzoic acid. Dr Sindelar is thus highly competent to evaluate our work which also involves 4-hydroxybenzoic acid analogues and their interaction with coenzyme Q metabolism.

Makoto Kawamukai Ph.D.
Professor, Shimane University
kawamuka@life.shimane-u.ac.jp
Prof. Kawamukai works on coenzyme Q biosynthesis in the yeast S. pombe. The CoQ biosynthetic pathway in this organism is very close from the ones present in S. cerevisiae and mammals. Prof. Kawamukai has a long experience with expression of human genes in yeast system and will be particularly competent to judge the part of our work that involves the expression of the human ferredoxins 1 and 2.

Gustav Dallner Ph.D.
Professor, Department of Biochemistry and Biophysics, The Arrhenius Laboratories, Stockholm
Gustav.Dallner@dbb.su.se
Prof. Dallner is reknown biochemist who has worked on coenzyme Q biosynthesis and functions for a long time. Prof. Dallner's expertise on CoQ indicates him as a potential reviewer for this paper.

Opposed Reviewers:

Dr. Fabien PIERREL
Laboratoire de Chimie et Biologie des Métaux
UMR5249 UJF-CNRS-CEA
CEA Grenoble - iRTSV Bât K'
17 rue des martyrs
38054 Grenoble Cedex 9
France

Tél : 04 38 78 91 10
Fax : 04 38 78 91 24
E-mail : fabien.pierrel@cea.fr

June 27th 2011

Dear Dr Kostic,

We are very happy that the 4 reviewers commented positively on our manuscript "**Coenzyme Q biosynthesis: Coq6 catalyzes the C5-hydroxylation reaction and substrate analogues rescue Coq6 deficiency"** by Mohammad Ozeir, Ulrich Mühlenhoff, Holger Webert, Roland Lill, Marc Fontecave, and Fabien Pierrel. We now submit a revised version of this manuscript "**Coenzyme Q biosynthesis: Coq6 is required for the C5-hydroxylation reaction and substrate analogues rescue Coq6 deficiency**" which takes into consideration the points raised by the reviewers.

In addition to all the requested modifications detailed in the "response to reviewers" file, we improved the completeness of the data set presented in fig 4D by including additional controls (cells transformed with a vector and Yah1-complemented cells with or without vanillic acid). The mitochondrial preparations used in these experiments were of better quality than the ones used to generate the data of the original submission explaining that the succinate dehydrogenase (SDH) activity values are now higher. Our original result that vanillic acid improved SDH-cyt c activity in Yah1-depleted cells expressing Fdx2 is completely reproduced in this new data set. Therefore, our original interpretation is reinforced.

Overall, we have modified the text of the manuscript as presented in the "response to reviewers" file and Figure 2, 3, 4, S1 and S3 have also been modified according to reviewers' comments.

We are confident that this revised manuscript is now suitable for publication in Chemistry and Biology and thank you for your consideration to our manuscript.

Sincerely yours

Dr. Fabien PIERREL

Dr. Fabien PIERREL
Laboratoire de Chimie et Biologie des Métaux
UMR5249 UJF-CNRS-CEA
CEA Grenoble - iRTSV Bât K'
17 rue des martyrs
38054 Grenoble Cedex 9
France

Tél : 04 38 78 91 10
Fax : 04 38 78 91 24
E-mail : fabien.pierrel@cea.fr

June 27th 2011

We are very happy that the 4 reviewers commented positively on our manuscript "**Coenzyme Q biosynthesis: Coq6 catalyzes the C5-hydroxylation reaction and substrate analogues rescue Coq6 deficiency"** by Mohammad Ozeir, Ulrich Mühlenhoff, Holger Webert, Roland Lill, Marc Fontecave, and Fabien Pierrel. We now submit a revised version of this manuscript "**Coenzyme Q biosynthesis: Coq6 is required for the C5-hydroxylation reaction and substrate analogues rescue Coq6 deficiency**" which takes into consideration the points raised by the reviewers.

For reviewer 1 :

1) The title has been revised.

2) We have characterized in our previous publication (Chem. Biol. (2010), 17: 449-459) the 4-AP and 4-HP intermediates accumulated in Yah1 and Arh1 depleted cells. Because of the limited amount of compounds accumulated by the *coq6* mutant strains, we did not repeat the NMR analysis. However, we provide irrefutable evidences that the compounds accumulated by the *coq6* mutant strains are indeed identical to 4-AP and 4-HP which accumulate in Yah1 and Arh1 depleted cells. First, they have the same retention time and electrochemical properties than 4-AP and 4-HP. Second, we now show in Fig. S1B the UV-vis spectra of both compounds which are identical to the ones we previously published for 4-HP and 4-AP (Fig S2C and S3B). Third, we now mention in the text the monitoring of the compounds by mass spectrometry. The 518/122 and the 519 /123 transitions are characteristics of the carboxytropylium ions formed upon fragmentation of 4-AP and 4-HP respectively. The undistinguishable HPLC-ECD, UV-vis and mass spectrometry properties of 4-AP/4-HP and of the compounds accumulated by the Coq6-deficient strains prove that these compounds are identical.

3) While a scheme showing the proposed relationship between Yah1, Arh1 and Coq6 may be helpful to readers, we consider at this point that our proposal is still speculative and we do not want to "formalize" it by a scheme which may latter be taken as a fact by readers. We added one sentence in the discussion to further clarify our proposed relationship between Yah1, Arh1 and Coq6.

4) Our present experiments clearly establish that 4-AP and 4-HP do not transfer electrons in the respiratory chain since yeast strains accumulating these compounds can not grow on respiratory carbon sources (Fig 3A, B). In this sense, 4-AP and 4-HP may be considered non-functional. However, the possible function of these compounds in other established Q-dependent cellular functions like an antioxidant activity or an influence on the mitochondrial permeability pore has not been addressed yet but is certainly behind the scope of this manuscript. Whether 4-AP and 4-HP may be processed into Q, if the enzymatic deficiency that led to their accumulation is relieved, is not known either.

5) We have described in our previous publication (Chem. Biol. (2010), 17: 449-459) that endogenously synthesized pABA and 4-HB are limiting for Q biosynthesis. We again demonstrate this in fig. S1A by showing that biosynthesized Q levels are increased when exogenous pABA or 4-HB are added to the culture medium.

6) The figures in the supplemental section are now correctly identified.

For reviewer 2 :

1) Comment 2 from reviewer 3 recommends the opposite of comment 1 from reviewer 2, i.e. include supplemental figures into the main text. In this revised version, we have been careful to keep in the figures the data which are essential to the comprehension of the study while the supporting data have been included into the supplemental figures. We paid attention to clarify the presented chromatograms by either decreasing their numbers or increasing their contrast or increasing the spacing between them. In this regard, one chromatogram from fig 2A and one from fig 2B have been moved to Fig S1, in which the chromatograms are now displayed in two panels. One chromatogram from fig 3A has been moved to Fig S3, in which the chromatograms are now displayed in two panels.

We chose to keep in the main figures chromatograms from cells grown in pABA and 4-HB because 4-AP and 4-HP respectively are characteristic of an impaired C5-hydroxylation. As pointed in comment 4, 4-HP has a retention time very similar to DMQ whereas no other compound elutes close to 4-AP. In consequence, the accumulation of 4-AP which we can unambiguously identify based on its retention time, is diagnostic of a deficiency in the C5-hydroxylation and corroborates the accumulation of 4-HP (which elutes at a retention time close to that of DMQ) seen with cells grown in the presence of 4-HB.

2) Large variation of the Q/DMQ ratio throughout the growth of S. cerevisiae has been described (Cell Mol Life Sci 2009,66,173-186). The data previously presented in Fig. S1 showed an equal abundance of Q and DMQ for the WT cells which were harvested in exponential growth. We repeated the experiment and analysed the quinone content of cells harvested in stationary phase. The

new chromatograms are displayed in Fig. S1A and are similar to the other chromatograms of WT cells (again harvested in stationary phase) presented in other figures.

3) The Δcoq6/Coq8 strain now presented in Fig S1C accumulates 4-HP and no 4-AP because when no exogenous precursor of Q is supplied in the growth medium (pABA or 4-HB) then endogenous 4-HB seems to be the preferred substrate over pABA. This statement has been included in the manuscript.

4) When both 4-HP and DMQ are formed (fig 2C, 3A, S3C), the separation although not optimal is visible. We tried to improve the separation by varying the solvents (ethanol, mixture of isopropanol and acetonitrile) but we could not define conditions to better separate all the compounds analyzed (4-AP, 4-HP, DMQ6, Q6, N-DMQ6 (see comment 6 for reviewer 3)). As discussed in comment 1, the accumulation of 4-AP in cells grown in the presence of pABA corroborates the accumulation of 4-HP seen with cells grown in the presence of 4-HB. Concomitant accumulation of 4-HP and DMQ in Fig. 2C results from a partial complementation of the C5-hydroxylation deficiency. In fig 3A and S3C, 4-HP is formed by the endogenous 4-HB entering the Q pathway whereas DMQ is formed from the incomplete transformation of VA into Q_6.

5) Some repetitions and references have been deleted.

For reviewer 3 :

1) We changed yeast into *S. cerevisiae* where appropriate.

2) See comment 1 for reviewer 2.

3) The efficient restoration of Q biosynthesis by vanillic acid in the flx1 mutant (Fig S3D), implicates that only the C5-hydroxylation depends on FAD and therefore excludes that an unknown FAD-dependent protein could be required for another step of Q biosynthesis. Since our present work demonstrates that Coq6 is required for this C5-hydroxylation reaction and because Coq6 has been predicted to be a flavin-dependent monooxygenase, we feel that it is reasonable to assume that the FAD deficiency caused by the deletion of flx1 affects Coq6 activity and that Coq6 is an FAD-dependent protein. However, we agree with reviewer 3 that we do not have a biochemical proof that Coq6 is FAD-dependent and we took care of not making this statement in the text. In addition, based on the crystal structure of para-hydroxybenzoate hydroxylase (PobA) discussed in Protein Science 1997, 6, 2454-2458, the three conserved residues that we mutated in Coq6 are likely involved in FAD and NADPH binding as now stated in the text (the corresponding references were added). We are working on Coq6 heterologous expression and purification but so far our attempts resulted in obtaining an insoluble protein. In consequence, we are not able to address whether Coq6 is a FAD-binding protein but as discussed above, our study highly suggests this by clearly showing that Coq6 is required for the C5-hydroxylation and that the C5-hydroxylation is dependent on FAD.

4) We now show the requested data in Fig. 2C

5) See comment 2 for reviewer 2.

6) The peak corresponds to 4-aminodemethoxyquinone (N-DMQ) and forms in Yah1-depleted cells only when pABA is added to the growth medium. The incomplete shut-down of Yah1 results in formation of N-DMQ and of some residual Q as seen in Fig. 4B and S4. We are currently working on a collaborative manuscript in which a particular *coq* mutant accumulates greater amounts of N-DMQ than Yah1-depleted cells. In this future manuscript, the full characterization of N-DMQ will be detailed and its formation in Yah1-depleted cells will be discussed.

7) Our previous study (Chem. Biol. (2010), 17: 449-459) demonstrated the essentiality of Arh1 for the C5-hydroxylation reaction. We have modified the discussion to clarify this point.

8) The review by Kawamukai is now cited.

For reviewer 4 :

We corrected our statement and simply discuss the potential advantage of using hydrophilic substrate analogues like vanillic acid instead of Q supplementation in some primary Q deficiencies.

In addition to all these requested modifications, we improved the completeness of the data set presented in fig 4D by including additional controls (cells transformed with a vector and Yah1-complemented cells with or without vanillic acid). The mitochondrial preparations used in these experiments were of better quality than the ones used to generate the data of the original submission explaining that the succinate dehydrogenase (SDH) activity values are now higher. Our original result that vanillic acid improved SDH-cyt c activity in Yah1-depleted cells expressing Fdx2 is completely reproduced in this new data set.

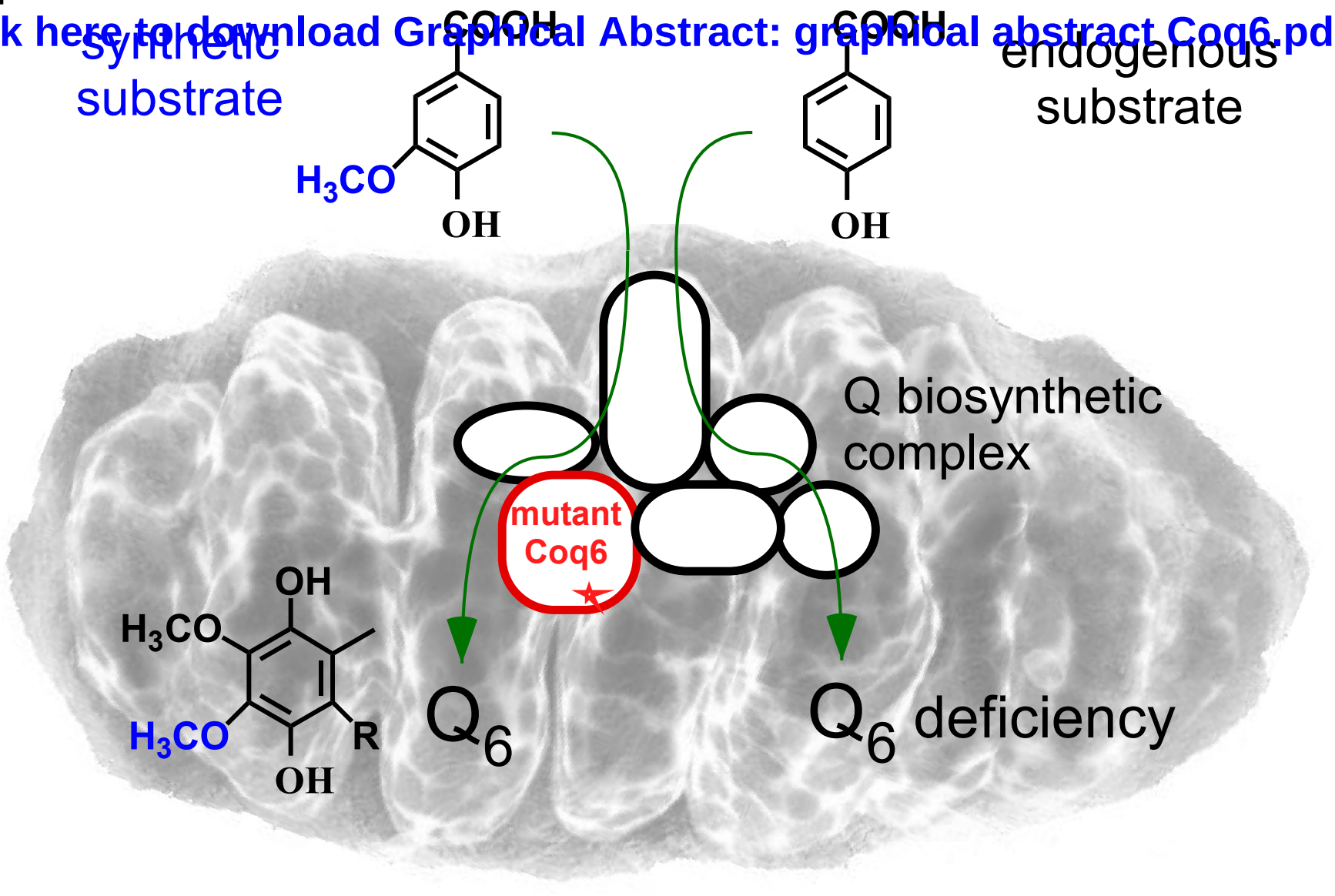
Overall, we have modified the text of the manuscript as presented above and Figure 2, 3, 4, S1 and S3 have also been modified according to reviewers' comments.

Sincerely yours

Dr. Fabien PIERREL

Graphical Abstract

Click here to download Graphical Abstract: graphical abstract Coq6.pdf



Coenzyme Q biosynthesis: Coq6 is required for the C5-hydroxylation reaction and substrate analogues rescue Coq6 deficiency

Mohammad Ozeir[1], Ulrich Mühlenhoff[2], Holger Webert[2], Roland Lill[2], Marc Fontecave[1,3], and Fabien Pierrel[1*]

[1] Laboratoire de Chimie et Biologie des Métaux; UMR5249 CNRS-CEA-UJF, CEA Grenoble, 17 rue des Martyrs, 38054 Grenoble Cedex 9, France

[2] Institut für Zytobiologie und Zytopathologie, Philipps-Universität Marburg, Robert-Koch-Strasse 6, 35032 Marburg, Germany

[3] Collège de France, 11 place Marcellin-Berthelot, 75005 Paris, France

[*] Contact
Laboratoire de Chimie et Biologie des Métaux
iRTSV, Bat K', P272
CEA Grenoble
17 rue des Martyrs
38054 Grenoble Cedex 9
France

Phone: +33-4-38-78-91-10
Fax: +33-4-38-78-91-24
E-mail: fabien.pierrel@cea.fr

Running Title: Substrate analogues bypass CoQ biosynthetic defect

Summary

Coenzyme Q (Q), an essential component of eukaryotic cells, is synthesized by several enzymes from the precursor 4-hydroxybenzoic acid. Mutations in six of the Q biosynthesis genes cause diseases that can sometimes be ameliorated by oral Q supplementation. We establish here that Coq6, a predicted flavin-dependent monooxygenase, is involved exclusively in the C5-hydroxylation reaction. In an unusual way, the ferredoxin Yah1 and the ferredoxin reductase Arh1 may be the in vivo source of electrons for Coq6. We also show that hydroxylated analogues of 4-hydroxybenzoic acid, such as vanillic acid or 3,4-dihydroxybenzoic acid, restore Q biosynthesis and respiration in a *Saccharomyces cerevisiae coq6* mutant. Our results demonstrate that appropriate analogues of 4-hydroxybenzoic acid can bypass a deficient Q biosynthetic enzyme and might be considered for the treatment of some primary Q deficiencies.

Highlights

- Inactivation of Coq6 abrogates the C5-hydroxylation in coenzyme Q biosynthesis
- Vanillic acid restores Q biosynthesis and respiration in *coq6 S. cerevisiae* mutants
- 4-hydroxybenzoic acid analogues bypass a deficient *S. cerevisiae* Q biosynthetic step

Introduction

Coenzyme Q or ubiquinone (Q) is a redox active lipid present in most organisms and in all tissues of multicellular eukaryotes where it shuttles electrons from complex I and II to complex III of the mitochondrial respiratory chain and acts as an important lipid-soluble antioxidant. Moreover, Q participates in the control of the mitochondrial membrane transition pore and functions with uncoupling proteins in the mitochondrial inner membrane (Bentinger et al., 2010). Q contains a polyprenyl tail with six isopentenyl units in *Saccharomyces cerevisiae* (Q_6) and ten in human (Q_{10}) (Kawamukai, 2009) (Figure 1). In *S. cerevisiae*, the biosynthesis of Q is accomplished by multiple conserved mitochondrial matrix enzymes (termed Coq1-Coq9) some of which are assembled in a large Q biosynthetic complex associated with the inner membrane (Tran and Clarke, 2007). Mutations affecting five genes involved in Q biosynthesis have been described and result in primary Q deficiencies that cause clinically heterogeneous diseases (Quinzii and Hirano, 2010). Oral Q_{10} supplementation of patients yields significant improvement in some cases (Quinzii and Hirano, 2010). Very recently, mutations in a sixth gene, *COQ6*, were shown to cause nephrotic syndrome with sensorineural deafness (Heeringa et al., 2011).

The initial stage of Q biosynthesis involves Coq2. In *S. cerevisiae*, Coq2 prenylates 4-hydroxybenzoic acid (4-HB) or the newly identified precursor para-aminobenzoic acid (pABA) to yield 3-hexaprenyl-4-hydroxybenzoic acid (HHB) and 3-hexaprenyl-4-aminobenzoic acid (HAB), respectively (Figure 1) (Marbois et al., 2010; Pierrel et al., 2010). The C4-amine originating from pABA is subsequently converted into an hydroxyl at an unidentified step of Q_6 biosynthesis (Figure 1). *S. cerevisiae* cells depleted for either the mitochondrial ferredoxin Yah1 or the mitochondrial ferredoxin reductase Arh1 synthesize almost no Q_6 and accumulate 3-hexaprenyl-4-aminophenol (4-AP) upon culturing in the

presence of pABA or 3-hexaprenyl-4-hydroxyphenol (4-HP) in the presence of 4-HB (Figure 1) (Pierrel et al., 2010). This result established that Yah1 and Arh1 are absolutely required for the C5-hydroxylation step. Arh1 and Yah1 form a well established electron transfer complex necessary in *S. cerevisiae* for iron sulphur cluster (ISC) and heme A biosynthesis (Barros et al., 2002; Lange et al., 2000; Li et al., 2001). Yah1 has two human homologues, Fdx1 and Fdx2. Fdx1 functions in steroid biogenesis by transferring electrons to mitochondrial cytochrome P450 enzymes whereas Fdx2 transfers electrons for the biogenesis of heme A and ISC (Sheftel et al., 2010).

Coq6 is required for Q_6 biosynthesis in *Saccharomyces cerevisiae* (Gin et al., 2003) and, based on its amino acid sequence, has been predicted to belong to the class A flavoprotein monooxygenase family suggesting that it contains a FAD cofactor (van Berkel et al., 2006). Coq6 has been postulated to function in the C1- and/or the C5-hydroxylation reactions (Gin et al., 2003) (Figure 1). This ambiguity has not been resolved partly because a *S. cerevisiae* mutant lacking the entire *COQ6* gene (Δ*coq6*) accumulates only the early Q_6 biosynthetic intermediate, HHB when grown in the presence of 4-HB (Gin et al., 2003). In fact, the Δ*coq3-9* mutants all accumulate HHB because most Coq polypeptides (excluding Coq1, Coq2, Coq5 and Coq8) are interdependent for their stability within the Q biosynthetic complex (Hsieh et al., 2007). Indeed, the absence of a single Coq polypeptide causes the degradation of other Coq proteins (Tran and Clarke, 2007), thus preventing the biosynthesis of Q_6 intermediates diagnostic of the altered step in Δ*coq* mutants. Consequently, it has been difficult to elucidate the precise function of Coq4, Coq6 and Coq9. Coq8, a predicted protein kinase, is essential for Q_6 biosynthesis and participates in the phosphorylation of Coq3, Coq5 and Coq7 (Tauche et al., 2008; Xie et al., 2011). Overexpression of Coq8 has been shown to restore the levels of Coq3 and Coq4 in most Δ*coq* mutants (Zampol et al., 2010). In addition, overexpression of Coq8 in a Δ*coq7* strain causes the accumulation of demethoxyquinone

(DMQ_6) (Padilla et al., 2009), the substrate of the C6-hydroxylase, Coq7 (Stenmark et al., 2001) (Figure 1). This result implies that overexpression of Coq8 in the Δ*coq7* strain prevents the degradation of the Coq polypeptides involved in Q_6 biosynthetic steps upstream of Coq7. DMQ_6 also forms in a Δ*coq7* strain expressing the inactive allele *COQ7-E233K* even in the absence of Coq8 overexpression (Padilla et al., 2004). It therefore seems that single mutations in a given Coq protein have less impact on the integrity of the Q_6 biosynthetic complex than null mutations.

In the current study, we sought to define which hydroxylation step of Q_6 biosynthesis is dependent on Coq6. We show that a Δ*coq6* strain expressing inactive *COQ6* alleles or overexpressing Coq8 accumulates products of the Q_6 biosynthetic pathway which establish that the monooxygenase Coq6 is specifically required for the C5-hydroxylation reaction. The functional combination of Yah1, Arh1 and Coq6 in the C5-hydroxylation reaction is discussed. In addition, we demonstrate that hydroxylated forms of 4-HB like vanillic acid or 3,4-dihydroxybenzoic acid are able to restore Q_6 biosynthesis and respiration in a *S. cerevisiae* strain deficient for Coq6. This represents the first indication that the use of 4-HB analogues might be considered as a strategy to bypass defective steps in the Q biosynthetic pathway.

Results

Coq6 is required for the C5- but not the C1-hydroxylation reaction of Q biosynthesis.

Regular synthetic yeast growth medium contains pABA which is a precursor of Q_6 (Marbois et al., 2010; Pierrel et al., 2010). In order to control the nature of the precursors employed for Q_6 biosynthesis, we used a synthetic medium without pABA for all our experiments. Electrochemical detection (ECD) of cell lipid extracts separated by HPLC revealed that

addition of pABA or 4-HB to the growth medium increased the Q_6 and DMQ_6 content of *S. cerevisiae* (Figure S1A) consistent with our previous conclusion that endogenous 4-HB is limiting for Q_6 biosynthesis (Pierrel et al., 2010). Overexpression of Coq8 in the Δ*coq6* mutant strain led to an accumulation of 4-AP or 4-HP in cells cultured in the presence of pABA or 4-HB, respectively, whereas no products were formed in the absence of Coq8 overexpression (Figure 2A). Identities of 4-AP and 4-HP were established previously (Pierrel et al., 2010) and here confirmed by their retention time in HPLC chromatograms, their UV-vis spectra (Figure S1B) and the expected ion transitions (m/z: 518.3/122 for 4-AP and 519.3/123 for 4-HP) in mass spectrometry coupled to HPLC (data not shown). Without pABA or 4-HB supplementation of the growth medium, the Δ*coq6* strain overexpressing Coq8 accumulated 4-HP suggesting that endogenous 4-HB enters the Q biosynthetic pathway preferentially over endogenous pABA (Figure S1C). We also mutated residues in Coq6 (G202, G386, N388) selected on the basis of their conservation in other flavoprotein monooxygenases (Figure S2) in which they are implicated in the binding of NADPH and FAD, as shown in the case of the para-hydroxybenzoate hydroxylase PobA (Eppink et al., 1997; Palfey et al., 1994). As expected, a vector coding for Coq6 restored Q_6 biosynthesis in the Δ*coq6* strain whereas G202A and G386A-N388D mutant alleles of *COQ6* were inactive as proven by the absence of Q_6 and DMQ_6 (Figure 2B and Figure S1C). However, these mutants caused an accumulation of 4-AP in Δ*coq6* cells cultured in the presence of pABA, and of 4-HP upon growth in the presence of 4-HB (Figure 2B). Together, these results show that a Δ*coq6* strain either expressing a Coq6 protein inactivated by point mutations or overexpressing Coq8 accumulates 4-AP and 4-HP. This finding demonstrates that the C1- but not the C5-hydroxylation reaction is still catalyzed in the absence of Coq6 activity (Figure 1). Since Coq6 is a predicted FAD-dependent monooxygenase (Gin et al., 2003; van Berkel et al., 2006), we checked whether the C5-hydroxylation is dependent on the availability of FAD in

mitochondria. Deletion of the gene *FLX1* which codes for a mitochondrial FAD transporter results in decreased activity of FAD-dependent mitochondrial enzymes (Bafunno et al., 2004; Tzagoloff et al., 1996). The Δ*flx1* strain showed markedly diminished levels of Q_6 and accumulated 4-AP and 4-HP when cultured in the presence of pABA and 4-HB respectively (Figure 2C). Furthermore, we observed that overexpression of Fad1, the flavin adenine dinucleotide synthetase, restored Q_6 biosynthesis in the Δ*flx1* strain, however without completely abolishing the formation of 4-HP (Figure 2C). This result is in agreement with the overexpression of Fad1 being able to partially complement the mitochondrial FAD deficiency of a *flx1* mutant strain (Wu et al., 1995). Collectively, our results show that the C5-hydroxylation depends on mitochondrial FAD and that Coq6, a predicted FAD-dependent monooxygenase, is essential for the C5- but not for the C1-hydroxylation reaction of Q_6 biosynthesis.

Vanillic acid and 3,4-dihydroxybenzoic acid restore Q_6 biosynthesis in strains lacking Coq6 activity.

We tested whether hydroxylated forms of 4-HB like 3,4-dihydroxybenzoic acid (3,4-diHB) or vanillic acid (VA) (Figure S3A) may enter the Q_6 biosynthetic pathway and therefore bypass the C5-hydroxylation step which is deficient in *coq6* mutants. Addition of 3,4-diHB or VA to the growth medium restored Q_6 biosynthesis in a dose-dependent manner in the Δ*coq6* strain overexpressing Coq8 (Figure 3A and Figure S3B) or expressing the inactive Coq6 mutant G386A-N388D (Figure S3C). The Δ*coq6* strain containing only an empty vector synthesized no Q_6 from VA showing that the integrity of the Q biosynthetic complex is required to convert VA in Q_6 (Figure S3B). The Δ*coq6* strain overexpressing Coq8 or expressing Coq6-G386A-N388D was able to grow on respiratory carbon sources supplemented with 3,4-diHB

or VA, showing that Q_6 biosynthesized from these substrate analogues is physiologically functional (Figure 3B). When pABA or 4-HB were added in place of VA or 3,4-diHB, the cells were not able to grow on respiratory substrates (Figure 3B), thus showing that 4-AP or 4-HP are not competent for electron transfer in the respiratory chain. VA also restored Q_6 biosynthesis in the Δ*flx1* mutant to 70% of WT (Figure S3D).

Addition of increasing concentrations of 4-HB to the growth medium containing 1mM VA gradually decreased Q_6 levels and promoted the accumulation of 4-HP in the Δ*coq6* strain overexpressing Coq8 (Figure 3C). pABA had a similar impact on Q_6 levels at concentrations comparable to those of 4-HB (Figure S3E). Altogether our data show that exogenous pABA and 4-HB compete with VA to enter the Q_6 biosynthetic pathway at the prenylation step catalyzed by Coq2 and that prenylated VA is converted into Q_6, therefore bypassing the deficient C5-hydroxylation reaction in *coq6* mutant cells.

Coq6 and Yah1 are functionally coupled in the C5-hydroxylation reaction.

Our results show that the phenotypes of cells depleted for Yah1 (Pierrel et al., 2010) or deficient for Coq6 are strikingly similar with regard to Q_6 biosynthesis suggesting a mechanism in which Coq6 and Yah1 work together in the C5-hydroxylation step. The following experiments establish indeed that depletion of Yah1 directly impacts Coq6 activity. First, we demonstrate that Coq6 is stable when Yah1 is depleted. We constructed a Gal-*YAH1 COQ6-3HA* strain which contains a chromosomal insertion of a sequence coding for a triple hemagglutinin (3HA) epitope tag on the 3' end of *COQ6* and allows for regulated expression of Yah1. Indeed, the native *YAH1* promoter has been replaced by the *GAL1-10* promoter allowing for expression of Yah1 in a culture medium containing galactose and for depletion of Yah1 in a medium containing glucose (Lange et al., 2000). The Coq6-3HA protein was detected by immunoblotting at the expected size of 57 kDa (Figure 4A). Depletion of Yah1 by

culturing the Gal-*YAH1 COQ6-3HA* strain in the presence of glucose had no effect on the steady-state level of Coq6 (Figure 4A). The presence of Coq6 in Yah1-depleted cells is also confirmed by the observation that VA or 3,4-diHB restores Q_6 biosynthesis in the Yah1-depleted cells regardless of Coq8 overexpression (Figure S4A), thus showing that the Q biosynthetic complex is intact and therefore that Coq6 is present. Second, overexpression of Fad1 did not complement the Q_6 biosynthetic defect in Yah1-depleted cells (Figure S4B), suggesting that this defect does not result from a depletion of mitochondrial FAD. These data together show that Yah1 depletion, while having a negative effect on Q_6 biosynthesis, has no effect on Coq6 stability or mitochondrial FAD content.

Human Fdx2 was recently shown to complement the ISC biosynthetic defect of Yah1-depleted cells (Sheftel et al., 2010). Surprisingly, none of the two human homologues of Yah1, Fdx1 or Fdx2 complemented the Q_6 defect of Yah1-depleted cells as shown from the observation that the cells contained almost no Q_6 and furthermore accumulated 4-AP upon culture in the presence of pABA (Figure 4B). Growth of the Gal-*YAH1* strain on synthetic medium containing the respiratory carbon source lactate was limited unless it contained a plasmid carrying the *YAH1* gene (Figure 4C). With a plasmid expressing Fdx2, growth was restored upon addition of VA or 3,4-diHB to the lactate medium while addition of 4-HB was without any effect (Figure 4C). Our results thus suggest that, in the absence of VA or 3,4-diHB, Q_6 is limiting for respiratory growth of the Gal-*YAH1* strain expressing Fdx2. This was confirmed by measuring the in vitro activity of succinate dehydrogenase (SDH), a multi-protein complex which contains three ISC and one FAD, all required for activity. As expected from the complementation of the ISC biosynthetic defect of Yah1-depleted cells by Fdx2 (Sheftel et al., 2010), Fdx2 significantly restored reduction of the chemical dichlorophenol-indophenol (DPIP) by SDH in Yah1-depleted cells, although not to WT levels (Figure 4D). Q_6 is required to transfer electrons from SDH to the cytochrome bc_1 complex which reduces

cytochrome c (cyt c). Fdx2 restored SDH-cyt c reductase activity in Yah1-depleted cells only when VA was added to the culture medium (Figure 4D). This result shows that the limiting factor in transferring electrons from succinate to cyt c in Yah1-depleted cells expressing human Fdx2 is Q_6, the level of which is restored by addition of VA to the growth medium (Figure S4A).

Discussion

Our current work addresses the precise functional role of the Coq6 monooxygenase in the hydroxylation reactions of the Q biosynthetic pathway. As shown previously in the case of the Δ*coq7* strain (Padilla et al., 2004; Padilla et al., 2009), overexpression of Coq8 or expression of inactive alleles of *COQ6* allow for preservation of the integrity of the Q_6 biosynthetic complex in a Δ*coq6* strain. We show here for the first time that under these conditions, the Δ*coq6* strain grown in the presence of pABA or 4-HB accumulates significant amounts of 4-AP and 4-HP respectively. The accumulation of 4-AP and 4-HP is diagnostic of an impaired C5-hydroxylation but of a functional C1-hydroxylation. As a consequence, the predicted FAD-dependent monooxygenase Coq6 is required for the C5-hydroxylation but not for the C1-hydroxylation, definitively resolving the uncertainty regarding which hydroxylation reaction is catalyzed by Coq6. As a result, the C1-hydroxylase is still unknown (Figure 5) and is unlikely to be any of the Coq proteins identified to date because among these, only Coq6 and Coq7 display sequence homologies with monooxygenases and Coq7 participates exclusively in the C6-hydroxylation (Behan and Lippard, 2010; Padilla et al., 2004). Some experiments reported here suggest that the C1-hydroxylase is unlikely to be a FAD-dependent monooxygenase. A Δ*flx1* strain, characterized by impaired FAD-dependent mitochondrial activities (Bafunno et al., 2004; Tzagoloff et al., 1996), accumulates 4-AP and 4-HP indicating that the C1-hydroxylase is functional in this mutant (Figure 2C).

Furthermore, restoration of Q_6 biosynthesis in Δ*flx1* strain by VA (Figure S3D) is consistent with the C1-hydroxylation proceeding normally in the Δ*flx1* strain.

Our results show that VA and 3,4-diHB can be used as Q_6 precursors and thus restore Q_6 biosynthesis and respiration in Δ*coq6* cells overexpressing Coq8 or expressing the Coq6 inactive alleles (Figure 5). This proves that analogues of 4-HB added to the growth medium of *S. cerevisiae* can reach the mitochondrial matrix, where Q_6 biosynthesis takes place, and then enter the Q_6 biosynthetic pathway via prenylation by Coq2 (Figure 5). The strong impact of minor quantities of pABA or 4-HB observed on the levels of Q_6 synthesized from VA (Figure 3C and Figure S3E) may be explained by two non-exclusive hypotheses. VA may not be as efficiently transported to the mitochondrial matrix as pABA and 4-HB or Coq2 may have a higher affinity for pABA and 4-HB compared to VA. In any case, Coq2 has a broad substrate specificity in agreement with early *in vitro* studies which showed that mitochondrial preparations from rat heart and liver were able to prenylate VA and 3,4-diHB (Nambudiri et al., 1977). In fact, these analogues of 4-HB meet the structural requirements for prenylation by the polyprenyl transferase Coq2, i.e. an electron-donating substituent at position 4 of the aromatic ring combined with a carboxylic acid, a strong electron-attracting group, at position 1 (Alam et al., 1975). On the contrary, 4-nitrobenzoic acid which harbours the strong electron-attracting nitro group at position 4 inhibits Coq2 and as a consequence acts as an inhibitor of Q biosynthesis in mammalian cells cultures (Forsman et al., 2010). We would like to suggest that 4-HB analogues compatible with prenylation by Coq2 may also bypass deficient Q biosynthetic steps downstream of the C5-hydroxylation reaction. It is interesting to note that most mutations identified so far in humans to cause primary Q deficiency are not found in genes encoding enzymes that catalytically modify the prenylated aromatic ring except for *COQ6* (Heeringa et al., 2011). In fact they are found in (i) *PDSS1* and *PDSS2* (Lopez et al., 2006; Mollet et al., 2007), which are the relatives of *COQ1* catalyzing the

synthesis of the polyprenyl tail, (ii) the polyprenyl transferase *COQ2* (Mollet et al., 2007), (iii) *ADCK3*/*CABC1* (Lagier-Tourenne et al., 2008; Mollet et al., 2008), the *COQ8* homolog and (iv) *COQ9*, a gene with no specific function assigned (Duncan et al., 2009). Our results suggest that VA may be efficient at promoting Q_{10} biosynthesis in patients with *COQ6* mutations. Practically, as vanillin (3-methoxy-4-hydroxybenzaldehyde), a common non-toxic food additive, is converted in the liver to VA (Muskiet and Groen, 1979; Sayavongsaa, 2007), vanillin may represent an interesting therapeutic molecule to try in patients with *COQ6* mutations.

Finally, we previously demonstrated that Yah1 and Arh1 are essential for the C5-hydroxylation reaction (Pierrel et al., 2010). The similar impact on Q_6 biosynthesis of Yah1 or Arh1 depletion or Coq6 inactivation raises the question of how these three enzymes are functionally coupled. Here we provide preliminary data aiming at understanding this link. Yah1/Arh1 form a ferredoxin/ferredoxin reductase system that transfers electrons for different mitochondrial processes, in particular ISC assembly (Lill, 2009). The fact that Fdx2, a human homolog of Yah1, complements the ISC biosynthetic defect but not the Q_6 biosynthetic defect of Yah1-depleted cells excludes that Coq6 and thus Q_6 metabolism may indirectly be affected by an impairment of ISC biosynthesis. Coq6 is classified among Class A flavoprotein monooxygenases which have been described to contain a tightly bound FAD cofactor and to depend on NAD(P)H as a coenzyme for reduction of FAD (van Berkel et al., 2006). We checked whether inactivation of Yah1 could indirectly cause a depletion of mitochondrial NAD(P)H or FAD which would result in decreased Coq6 activity. Our data summarized below indicate that, in the absence of Yah1, the Coq6 polypeptide is not degraded, mitochondrial FAD and NAD(P)H are available, and yet Coq6 is unable to perform the C5-hydroxylation, implying that Yah1 itself plays a role in this reaction. First, the Coq6-3HA polypeptide is detected in Yah1-depleted cells. Second, a shortage of mitochondrial FAD, the

predicted cofactor of Coq6, is unlikely. Indeed, Yah1-depleted cells expressing Fdx2 are impaired for the C5-hydroxylation reaction but display significant SDH activity, known to depend on FAD. In addition, overexpression of *FAD1* restored Q_6 biosynthesis in the Δ*flx1* strain whereas it failed to do so in Yah1-depleted cells. Third, mitochondrial NAD(P)H levels may not be dramatically affected in Yah1-depleted cells expressing Fdx2 since the latter is able to assemble ISC, a process supported by mitochondrial NADPH (Pain et al., 2010). Also in support of this, Yah1-depleted cells are prototroph for arginine (data not shown), the biosynthesis of which requires mitochondrial NADPH produced by Pos5 via phosphorylation of mitochondrial NADH (Outten and Culotta, 2003). As a conclusion, if Coq6 was a classical class A flavoprotein monooxygenase catalyzing reduction of FAD by NAD(P)H, it should be active in Yah1-depleted cells since their levels of mitochondrial FAD and NAD(P)H are not compromised. Consequently, we end up with the hypothesis of an unusual mechanism in which the reducing power of NAD(P)H may transit via the Yah1/Arh1 system before reaching Coq6. The β-cyclohexenyl carotenoid epoxidase, another class A flavoprotein monooxygenase, has been shown to require a ferredoxin/ferredoxin reductase system for *in vitro* activity (Bouvier et al., 1996) and thus represents a precedent for such an electron transfer pathway from a ferredoxin to a flavin-monoxygenase. Unequivocal demonstration that Coq6 may also use an unconventional reducing system like Arh1/Yah1 will necessitate the development of an *in vitro* assay with purified proteins; a challenging task given that Coq6 likely interacts with several Coq polypeptides in the Q_6 biosynthetic complex (Marbois et al., 2005). Nonetheless, our study has unambiguously established that Coq6 is required exclusively for the C5-hydroxylation of Q_6 biosynthesis and that hydroxylated analogues of 4-HB can be used as precursors of Q_6, two results that significantly improve our understanding of the biosynthesis of this crucial coenzyme.

Significance

Coenzyme Q (Q) or ubiquinone, an important cellular antioxidant, is essential to electron transport chains and is required for several other cellular processes. Q biosynthesis requires at least eleven proteins in *S. cerevisiae* but the precise function of several of them is not known. Our work establishes that the predicted monoxygenase Coq6 is involved in the C5-hydroxylation reaction and that an unidentified monooxygenase catalyzes the C1-hydroxylation reaction. We further demonstrate the possibility to bypass a deficient Q biosynthetic step in *S. cerevisiae* by providing the defective chemical group within a synthetic 4-hydroxybenzoic acid analogue. Indeed, a *coq6* mutant impaired in the C5-hydroxylation reaction recovers Q_6 biosynthesis and respiration upon addition of two such analogues, 3,4-dihydroxybenzoic acid and vanillic acid. Primary Q_{10} deficiencies have been linked to mutations in six genes of the Q biosynthetic pathway and result in clinically heterogeneous diseases which, if diagnosed early, are improved by Q_{10} supplementation. However, the lipophilicity of Q_{10} may restrain its efficient transport to the mitochondrial inner membrane where Q_{10} functions in the respiratory chain (Quinzii and Hirano, 2010). Our work suggests that hydrophilic analogues of 4-hydroxybenzoic acid may restore Q_{10} biosynthesis in patients with some primary Q_{10} deficiencies by bypassing the altered biosynthetic step. The use of various 4-hydroxybenzoic acid analogues will also contribute to characterize the biosynthetic step(s) blocked in some *S. cerevisiae coq* mutants and will thus help to identify the molecular function of Coq proteins with unknown function. Our work illustrates the importance of a molecular understanding of the Q biosynthetic pathway and warrants the identification of yet unidentified proteins that participates in Q biosynthesis, in particular the monooxygenase responsible for the C1-hydroxylation reaction.

Experimental Procedures

Yeast strains and culture conditions: *S. cerevisiae* strains used in this study are listed in Table S1. *S. cerevisiae* strains were transformed using lithium acetate. A 3HA epitope tag was inserted on the 3' end of *COQ6* ORF by PCR as described previously (Longtine et al., 1998) to create the *COQ6-3HA* strain. This strain was crossed with the Gal-*YAH1* strain to isolate the Gal-*YAH1 COQ6-3HA* strain by selecting the corresponding markers after tetrad dissection. YNB without pABA and folate was from MP Biomedicals. Rich YP medium was prepared as described (Sherman, 2002). Glucose, galactose or lactate-glycerol were used at 2%. The Gal-*YAH1* strain was maintained and precultured on galactose medium. Depletion of Yah1 was accomplished by diluting 200 fold the preculture into glucose containing medium and growing the cells for 18 hours at 30°C.

Plasmids: *COQ6* ORF was cloned into pRS416 under the control of the *MET25* promoter and the *CYC1* terminator using XhoI and XbaI (Mumberg et al., 1994). This vector served as a template to generate the G202V and G386A-N388D Coq6 mutants by site directed mutagenesis. *FAD1* was cloned with its own promoter (370 bp) and terminator (210 bp) into pRS423 using EcoRI and XhoI. Fdx1 and Fdx2 expressing plasmids have been described (Sheftel et al., 2010) and *COQ8* cloned in pFL44 was a kind gift from Dr. Geneviève Dujardin. Sequencing was used to confirm cloning products in all created vectors.

Miscellaneous biochemical analysis: Isolation of mitochondria and immunostaining were performed as described (Diekert et al., 2001; Harlow and Lane, 1988). Cellular lipid extraction after addition of the Q_4 standard and quantification of electroactive compounds by HPLC-ECD with a 5011A analytical cell (E1, –420 mV; E2, +380 mV) were as described (Pierrel et al., 2010). Hydroquinones present in samples were oxidized with a pre-column

5020 guard cell set in oxidizing mode (E, +650 mV). Mitochondrial enzymatic activities were measured as previously described (Pierik et al., 2009).

Acknowledgments

This work was supported in part by the Région Rhône-Alpes program CIBLE 2009 (to F.P.).

UM and RL acknowledge generous support from Deutsche Forschungsgemeinschaft (SFB 593 and TR1), von Behring-Röntgen Stiftung, LOEWE program of state Hessen, Max-Planck Gesellschaft, and Fonds der chemischen Industrie. We thank Dr Sandrine Ollagnier and Dr Gustav Berggren for critical reading of the manuscript.

References

Alam, S.S., Nambudiri, A.M., and Rudney, H. (1975). 4-Hydroxybenzoate: polyprenyl transferase and the prenylation of 4-aminobenzoate in mammalian tissues. Arch. Biochem. Biophys. 171, 183-190.

Bafunno, V., Giancaspero, T.A., Brizio, C., Bufano, D., Passarella, S., Boles, E., and Barile, M. (2004). Riboflavin uptake and FAD synthesis in Saccharomyces cerevisiae mitochondria: involvement of the Flx1p carrier in FAD export. J. Biol. Chem. 279, 95-102.

Barros, M.H., Nobrega, F.G., and Tzagoloff, A. (2002). Mitochondrial ferredoxin is required for heme A synthesis in Saccharomyces cerevisiae. J. Biol. Chem. 277, 9997-10002.

Behan, R.K., and Lippard, S.J. (2010). The aging-associated enzyme CLK-1 is a member of the carboxylate-bridged diiron family of proteins. Biochemistry 49, 9679-9681.

Bentinger, M., Tekle, M., and Dallner, G. (2010). Coenzyme Q-biosynthesis and functions. Biochem. Biophys. Res. Commun. 396, 74-79.

Bouvier, F., d'Harlingue, A., Hugueney, P., Marin, E., Marion-Poll, A., and Camara, B. (1996). Xanthophyll biosynthesis - Cloning, expression, functional reconstitution, and regulation of beta-cyclohexenyl carotenoid epoxidase from pepper (Capsicum annuum). J. Biol. Chem. 271, 28861-28867.

Diekert, K., de Kroon, A.I., Kispal, G., and Lill, R. (2001). Isolation and subfractionation of mitochondria from the yeast Saccharomyces cerevisiae. Methods Cell Biol 65, 37-51.

Duncan, A.J., Bitner-Glindzicz, M., Meunier, B., Costello, H., Hargreaves, I.P., Lopez, L.C., Hirano, M., Quinzii, C.M., Sadowski, M.I., Hardy, J., Singleton, A., Clayton, P.T., and Rahman, S. (2009). A Nonsense Mutation in COQ9 Causes Autosomal-Recessive Neonatal-Onset Primary Coenzyme Q(10) Deficiency: A Potentially Treatable Form of Mitochondrial Disease. Am. J. Hum. Genet. 84, 558-566.

Eppink, M.H., Schreuder, H.A., and Van Berkel, W.J. (1997). Identification of a novel conserved sequence motif in flavoprotein hydroxylases with a putative dual function in FAD/NAD(P)H binding. Protein Sci. 6, 2454-2458.

Forsman, U., Sjoberg, M., Turunen, M., and Sindelar, P.J. (2010). 4-Nitrobenzoate inhibits coenzyme Q biosynthesis in mammalian cell cultures. Nat. Chem. Biol. 6, 515-517.

Gin, P., Hsu, A.Y., Rothman, S.C., Jonassen, T., Lee, P.T., Tzagoloff, A., and Clarke, C.F. (2003). The Saccharomyces cerevisiae COQ6 gene encodes a mitochondrial flavin-dependent monooxygenase required for coenzyme Q biosynthesis. J. Biol. Chem. 278, 25308-25316.

Harlow, E., and Lane, D. (1988). (Cold Spring Harbour, NY: Cold Spring Harbour Laboratory Press).

Heeringa, S.F., Chernin, G., Chaki, M., Zhou, W., Sloan, A.J., Ji, Z., Xie, L.X., Salviati, L., Hurd, T.W., Vega-Warner, V., Killen, P.D., Raphael, Y., Ashraf, S., Ovunc, B., Schoeb, D.S., McLaughlin, H.M., Airik, R., Vlangos, C.N., Gbadegesin, R., Hinkes, B., Saisawat, P., Trevisson, E., Doimo, M., Casarin, A., Pertegato, V., Giorgi, G., Prokisch, H., Rotig, A., Nurnberg, G., Becker, C., Wang, S., Ozaltin, F., Topaloglu, R., Bakkaloglu, A., Bakkaloglu, S.A., Muller, D., Beissert, A., Mir, S., Berdeli, A., Varpizen, S., Zenker, M., Matejas, V., Santos-Ocana, C., Navas, P., Kusakabe, T., Kispert, A., Akman, S., Soliman, N.A., Krick, S., Mundel, P., Reiser, J., Nurnberg, P., Clarke, C.F., Wiggins, R.C., Faul, C., and Hildebrandt, F. (2011). COQ6 mutations in human patients produce nephrotic syndrome with sensorineural deafness. J. Clin. Invest. 121, 2013-2024.

Hsieh, E.J., Gin, P., Gulmezian, M., Tran, U.C., Saiki, R., Marbois, B.N., and Clarke, C.F. (2007). Saccharomyces cerevisiae Coq9 polypeptide is a subunit of the mitochondrial coenzyme Q biosynthetic complex. Arch. Biochem. Biophys. 463, 19-26.

Kawamukai, M. (2009). Biosynthesis and bioproduction of coenzyme Q10 by yeasts and other organisms. Biotechnol. Appl. Biochem. 53, 217-226.

Lange, H., Kaut, A., Kispal, G., and Lill, R. (2000). A mitochondrial ferredoxin is essential for biogenesis of cellular iron-sulfur proteins. Proc. Natl. Acad. Sci. U. S. A. 97, 1050-1055.

Li, J., Saxena, S., Pain, D., and Dancis, A. (2001). Adrenodoxin reductase homolog (Arh1p) of yeast mitochondria required for iron homeostasis. J. Biol. Chem. 276, 1503-1509.

Lill, R. (2009). Function and biogenesis of iron-sulphur proteins. Nature 460, 831-838.

Longtine, M.S., McKenzie, A., 3rd, Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast 14, 953-961.

Marbois, B., Gin, P., Faull, K.F., Poon, W.W., Lee, P.T., Strahan, J., Shepherd, J.N., and Clarke, C.F. (2005). Coq3 and Coq4 define a polypeptide complex in yeast mitochondria for the biosynthesis of coenzyme Q. J. Biol. Chem. 280, 20231-20238.

Marbois, B., Xie, L.X., Choi, S., Hirano, K., Hyman, K., and Clarke, C.F. (2010). para-Aminobenzoic Acid Is a Precursor in Coenzyme Q(6) Biosynthesis in Saccharomyces cerevisiae. J. Biol. Chem. 285, 27827-27838.

Mollet, J., Giurgea, I., Schlemmer, D., Dallner, G., Chretien, D., Delahodde, A., Bacq, D., de Lonlay, P., Munnich, A., and Rotig, A. (2007). Prenyldiphosphate synthase, subunit 1 (PDSS1) and OH-benzoate polyprenyltransferase (COQ2) mutations in ubiquinone deficiency and oxidative phosphorylation disorders. J. Clin. Invest. 117, 765-772.

Mumberg, D., Muller, R., and Funk, M. (1994). Regulatable promoters of Saccharomyces cerevisiae: comparison of transcriptional activity and their use for heterologous expression. Nucleic Acids Res. 22, 5767-5768.

Muskiet, F.A.J., and Groen, A. (1979). Urinary-excretion of conjugated homovanillic-acid, 3,4-dihydroxyphenylacetic acid, para-hydroxyphenylacetic acid, and vanillic acid by persons on their usual diet and patients with neuro-blastoma. Clinical Chemistry 25, 1281-1284.

Nambudiri, A.M.D., Brockman, D., Alam, S.S., and Rudney, H. (1977). Alternate routes for ubiquinone biosynthesis in rats. Biochem. Biophys. Res. Commun. 76, 282-288.

Outten, C.E., and Culotta, V.C. (2003). A novel NADH kinase is the mitochondrial source of NADPH in Saccharomyces cerevisiae. EMBO J. 22, 2015-2024.

Padilla, S., Jonassen, T., Jimenez-Hidalgo, M.A., Fernandez-Ayala, D.J., Lopez-Lluch, G., Marbois, B., Navas, P., Clarke, C.F., and Santos-Ocana, C. (2004). Demethoxy-Q, an intermediate of coenzyme Q biosynthesis, fails to support respiration in Saccharomyces cerevisiae and lacks antioxidant activity. J. Biol. Chem. 279, 25995-26004.

Padilla, S., Tran, U.C., Jimenez-Hidalgo, M., Lopez-Martin, J.M., Martin-Montalvo, A., Clarke, C.F., Navas, P., and Santos-Ocana, C. (2009). Hydroxylation of demethoxy-Q6 constitutes a control point in yeast coenzyme Q6 biosynthesis. Cell. Mol. Life Sci. 66, 173-186.

Pain, J., Balamurali, M.M., Dancis, A., and Pain, D. (2010). Mitochondrial NADH Kinase, Pos5p, Is Required for Efficient Iron-Sulfur Cluster Biogenesis in Saccharomyces cerevisiae. J. Biol. Chem. 285, 39409-39424.

Palfey, B.A., Entsch, B., Ballou, D.P., and Massey, V. (1994). Changes in the catalytic properties of p-hydroxybenzoate hydroxylase caused by the mutation Asn300Asp. Biochemistry 33, 1545-1554.

Pierik, A.J., Netz, D.J., and Lill, R. (2009). Analysis of iron-sulfur protein maturation in eukaryotes. Nat. Protoc. 4, 753-766.

Pierrel, F., Hamelin, O., Douki, T., Kieffer-Jaquinod, S., Muhlenhoff, U., Ozeir, M., Lill, R., and Fontecave, M. (2010). Involvement of Mitochondrial Ferredoxin and Para-Aminobenzoic Acid in Yeast Coenzyme Q Biosynthesis. Chem. Biol. 17, 449-459.

Quinzii, C.M., and Hirano, M. (2010). Coenzyme Q and mitochondrial disease. Dev Disabil Res Rev 16, 183-188.

Sayavongsaa, P., Cooperb, M.L., Jacksona, E.M., Harrisa, L., Zieglerc, T.R., Hibbert, J.M. (2007). Vanillic acid excretion can be used to assess compliance with dietary supplements. European e-Journal of Clinical Nutrition and Metabolism 2, e134-e137.

Sheftel, A.D., Stehling, O., Pierik, A.J., Elsasser, H.P., Muhlenhoff, U., Webert, H., Hobler, A., Hannemann, F., Bernhardt, R., and Lill, R. (2010). Humans possess two mitochondrial ferredoxins, Fdx1 and Fdx2, with distinct roles in steroidogenesis, heme, and Fe/S cluster biosynthesis. Proc. Natl. Acad. Sci. U. S. A. 107, 11775-11780.

Sherman, F. (2002). Getting started with yeast. In Guide to Yeast Genetics and Molecular and Cell Biology, Pt B, Volume 350. (San Diego: Academic Press Inc), pp. 3-41.

Stenmark, P., Grunler, J., Mattsson, J., Sindelar, P.J., Nordlund, P., and Berthold, D.A. (2001). A new member of the family of di-iron carboxylate proteins. Coq7 (clk-1), a membrane-bound hydroxylase involved in ubiquinone biosynthesis. J. Biol. Chem. 276, 33297-33300.

Tran, U.C., and Clarke, C.F. (2007). Endogenous synthesis of coenzyme Q in eukaryotes. Mitochondrion 7 Suppl, S62-71.

Tzagoloff, A., Jang, J., Glerum, D.M., and Wu, M. (1996). FLX1 codes for a carrier protein involved in maintaining a proper balance of flavin nucleotides in yeast mitochondria. J. Biol. Chem. 271, 7392-7397.

van Berkel, W.J.H., Kamerbeek, N.M., and Fraaije, M.W. (2006). Flavoprotein monooxygenases, a diverse class of oxidative biocatalysts. J. Biotechnol. 124, 670-689.

Wu, M., Repetto, B., Glerum, D.M., and Tzagoloff, A. (1995). Cloning and characterization of FAD1, the structural gene for flavin adenine-dinucleotide synthetase of Saccharomyces cerevisiae. Mol. Cell. Biol. 15, 264-271.

Zampol, M.A., Busso, C., Gomes, F., Ferreira-Junior, J.R., Tzagoloff, A., and Barros, M.H. (2010). Over-expression of COQ10 in Saccharomyces cerevisiae inhibits mitochondrial respiration. Biochem. Biophys. Res. Commun. 402, 82-87.

Figure legends

Figure 1: Current model of the *S. cerevisiae* coenzyme Q biosynthetic pathway. The names of proteins (underlined) and intermediates (italicized) relevant to this study are for *S. cerevisiae*. The numbering of the aromatic carbon atoms used throughout this study is shown on HHB and Q_6. Prenylation of 4-hydroxybenzoic acid (4-HB) or para-aminobenzoic acid (pABA) by Coq2 yields 3-hexaprenyl-4-hydroxybenzoic acid (HHB) or 3-hexaprenyl-4-aminobenzoic acid (HAB). In subsequent reactions, R stands for the hexaprenyl tail and X designates NH_2 or OH. NH_2 is converted into OH prior to demethoxyubiquinone (DMQ_6) formation (Marbois et al., 2010; Pierrel et al., 2010). The three hydroxylation reactions necessary to yield Q_6 are shown in red and the potential implication of Coq6 in the C5- and/or the C1-hydroxylation reactions is indicated (Coq6?). In cells depleted for Yah1 or Arh1, the C5-hydroxylation is deficient which results in synthesis of 3-hexaprenyl-4-aminophenol (4-AP) from HAB and of 3-hexaprenyl-4-hydroxyphenol (4-HP) from HHB (green dashed arrows and green boxes) (Pierrel et al., 2010).

Figure 2: Coq6 is required for the C5- but not for the C1-hydroxylation of Q_6 biosynthesis. (A) WT cells or Δ*coq6* cells transformed with an empty vector (vec) or with an episomal vector coding for Coq8 were grown in 2% glucose synthetic medium without pABA containing or not 100 μM 4-hydroxybenzoic acid (4-HB) or 100 μM pABA. Lipid extracts of 1 mg of cells (WT), 2 mg of cells (/Coq8) or 8 mg of cells (/vec) were analyzed by HPLC-ECD. Chemical structures of 3-hexaprenyl-4-aminophenol (4-AP, eluting at 610 sec) and of 3-hexaprenyl-4-hydroxyphenol (4-HP, eluting at 810 sec) are displayed. The peaks corresponding to coenzyme Q_6 (Q_6), demethoxyquinone (DMQ_6) and to the Q_4 standard are indicated. (B) Δ*coq6* cells transformed with an empty vector (vec) or centromeric vectors coding either for Coq6 or Coq6-G386A-N388D were grown in glucose synthetic medium

without pABA containing 100 μM pABA or 100 μM 4-HB. Lipid extracts of 4 mg of cells were analyzed by HPLC-ECD. (C) WT and Δ*flx1* cells transformed or not with an episomal vector coding for Fad1 were grown in glucose synthetic medium without pABA supplemented or not with pABA or 4-HB at 100 μM. Lipid extracts of 4 mg of cells (Δ*flx1*) and 1 mg of WT were analyzed by HPLC-ECD.

Figure 3: Vanillic acid and 3,4-dihydroxybenzoic acid restore Q_6 biosynthesis and respiration in *coq6* mutant cells (A) Δ*coq6* cells transformed with an episomal vector coding for Coq8 were grown in 2% glucose synthetic medium without pABA containing the indicated concentrations of 4-HB, pABA or vanillic acid (VA). Lipid extracts of 4 mg of cells were analyzed by HPLC-ECD. (B) Δ*coq6* cells transformed either with an episomal vector coding for Coq8, an empty vector (vec) or with centromeric vectors coding for WT Coq6 or Coq6-G386A-N388D were grown in glucose synthetic medium for 24 hours and serial dilutions were spotted onto agar plates. The plates contained synthetic medium without pABA supplemented with 2% glucose (Glu) or 2% lactate-2% glycerol (LG) and either vanillic acid (VA), pABA or 3,4-dihydroxybenzoic acid (3,4-diHB). The plates were incubated for 2 days (Glu) or 4 days (LG) at 30°C. (C) Quantification of Q_6 and 4-HP formed in Δ*coq6* cells overexpressing Coq8 grown in glucose synthetic medium without pABA with 1mM VA and the indicated concentration of 4-HB. Error bars are standard deviation (n=2).

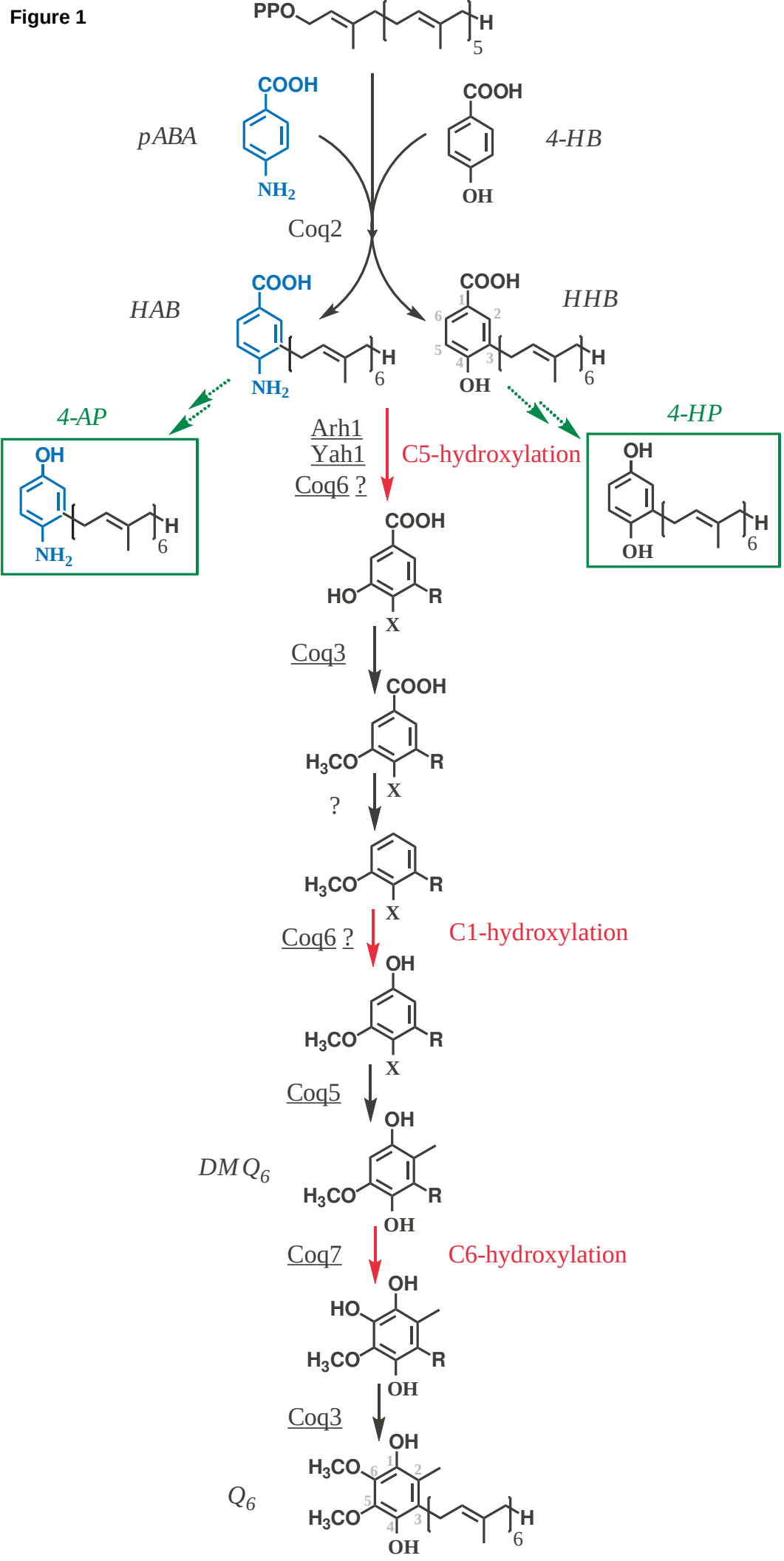
Figure 4: Q_6 is limiting for respiratory growth of Yah1-depleted cells expressing Fdx2. (A) Immunoblot of whole-cell lysates from WT, *COQ6-3HA* and Gal-*YAH1 COQ6-3HA* (two clones) cells grown in YP rich medium containing either 2% glucose (Glu) or 2% galactose (Gal). Coq6-3HA was detected at 57 kDa with an anti-HA antibody and as expected was

absent from extracts of the WT strain. Yah1 was detected around 16 kDa and Anc2, the major ADP/ATP carrier of the mitochondrial inner membrane, at 35kDa. (B) Gal-*YAH1* cells transformed with an empty vector (vec), a centromeric vector coding for Yah1 or episomal vectors coding for mitochondrially targeted human ferredoxin 1 or 2 (Fdx1 or Fdx2) were grown for 24 hours in synthetic medium supplemented with glucose and 1mM pABA. Lipid extracts of 4 mg of cells were analyzed by HPLC-ECD. (C) Same cells as in (B) were grown in glucose synthetic medium without pABA for 24 hours and serial dilutions were spotted onto agar plates. The plates contained synthetic medium without pABA supplemented at 2% with either glucose (Glu), galactose (Gal) or lactate (Lac) and either vanillic acid (VA), 3,4-dihydroxybenzoic acid (3,4-diHB) or 4-HB. The plates were incubated for 4 days at 30°C. (D) Gal-*YAH1* cells containing an empty vector (vec), a centromeric vector coding for Yah1 (Yah1) or an episomal vector coding for mitochondrially-targeted human ferredoxin 2 (Fdx2) were cultivated in glucose synthetic medium for 26 hours and finally in glucose synthetic medium without pABA in the presence or absence of 1 mM vanillic acid (VA) for 14 hours. Mitochondria were isolated and the enzyme activities of SDH (succinate to dichlorophenol-indophenol (DPIP)) and SDH in combination with complex III (succinate to cytochrome *c* (cyt c)) were determined and normalized to malate dehydrogenase activity (MDH). Error bars are standard deviation (n=3).

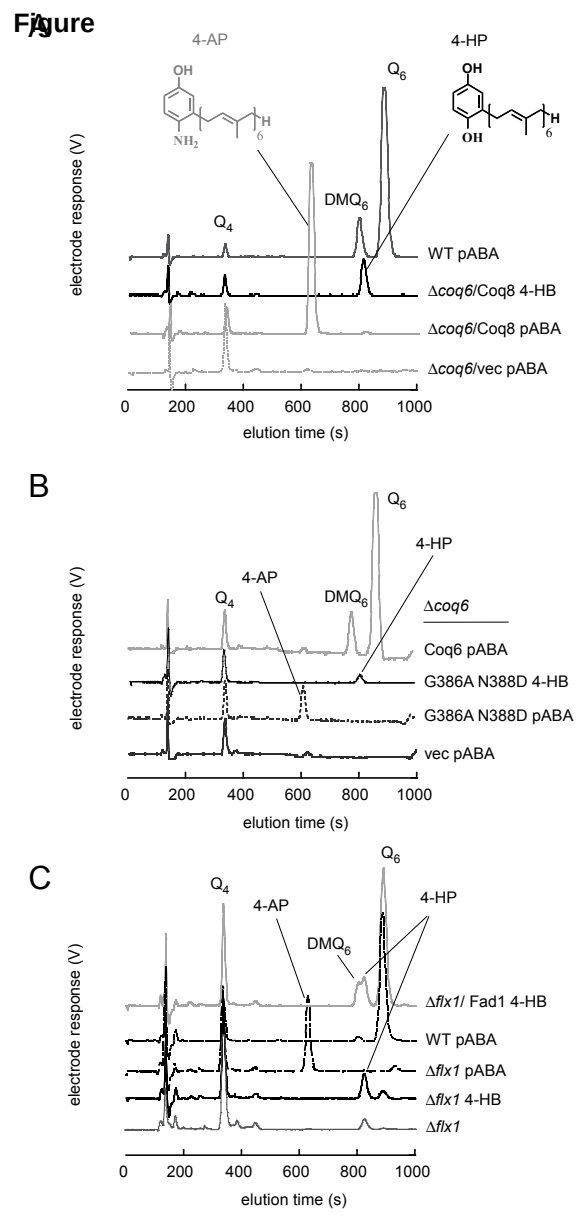
Figure 5: Coq6 is required for the C5-hydroxylation of Q_6 biosynthesis but Coq6 deficiency can be bypassed by using analogues of 4-HB. The pathway leading to Q_6 biosynthesis in WT *S. cerevisiae* cells is shown (above dashed line) with Coq6 implicated together with Yah1 and Arh1 in the C5-hydroxylation, whereas the C1-hydroxylation is catalyzed by an unidentified protein (?). In Yah1-depleted cells or in Δ*coq6* cells overexpressing either *COQ8* or an inactive *COQ6* allele (below dashed line), the C5-

hydroxylation does not take place (crossed arrow) but the C1-decarboxylation (dashed arrow) and the C1-hydroxylation proceed efficiently, leading to the accumulation of 4-HP when 4-HB is prenylated, or the accumulation of 4-AP when pABA is prenylated (blue). 3,4-diHB and VA contain an additional C5-hydroxyl (green) or C5-methoxyl (green) compared to 4-HB. 3,4-diHB and VA which correspond to the unprenylated products of the reactions catalyzed by Coq6 and Coq3 are prenylated *in vivo* by Coq2 and restore Q_6 biosynthesis in cells deficient for Coq6.

Figure 1

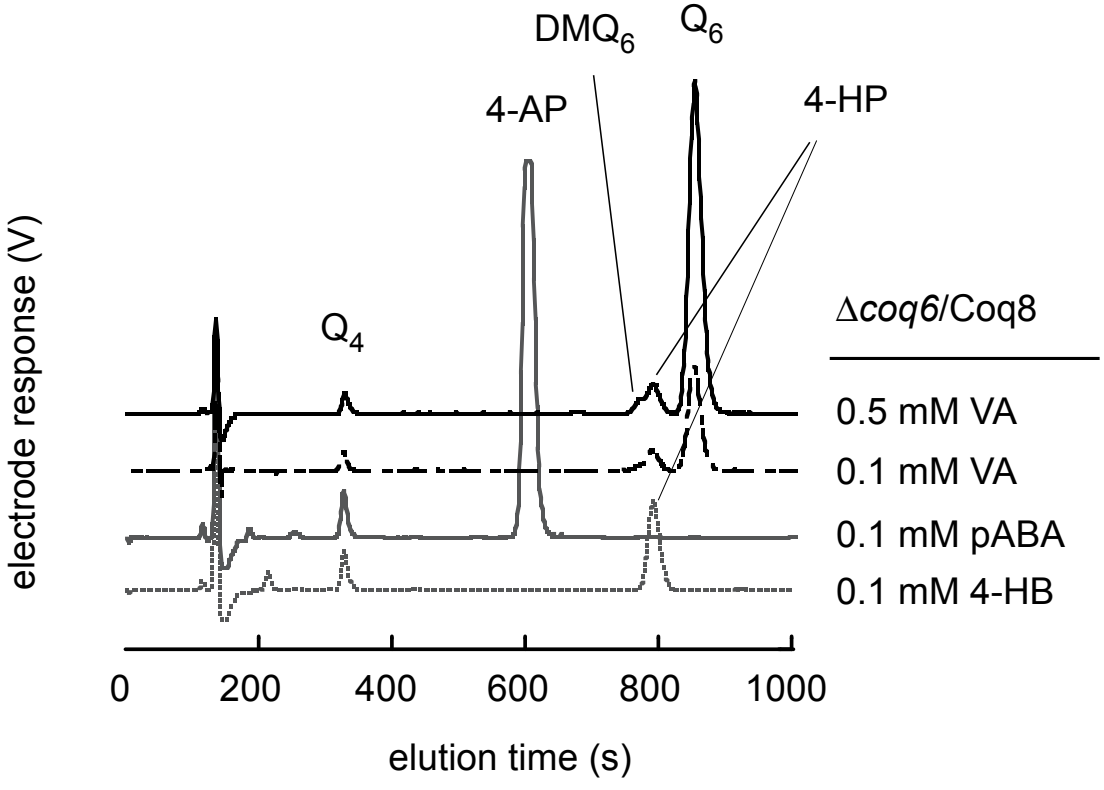

PPO
5
pABA
4-HB
COOH
NH2
OH
Coq2
HAB
HHB
6
4-AP
4-HP
Arh1
Yah1
Coq6 ?
C5-hydroxylation
HO
R
X
Coq3
H3CO
?
C1-hydroxylation
Coq5
DMQ6
Coq7
C6-hydroxylation
Q6

Figure

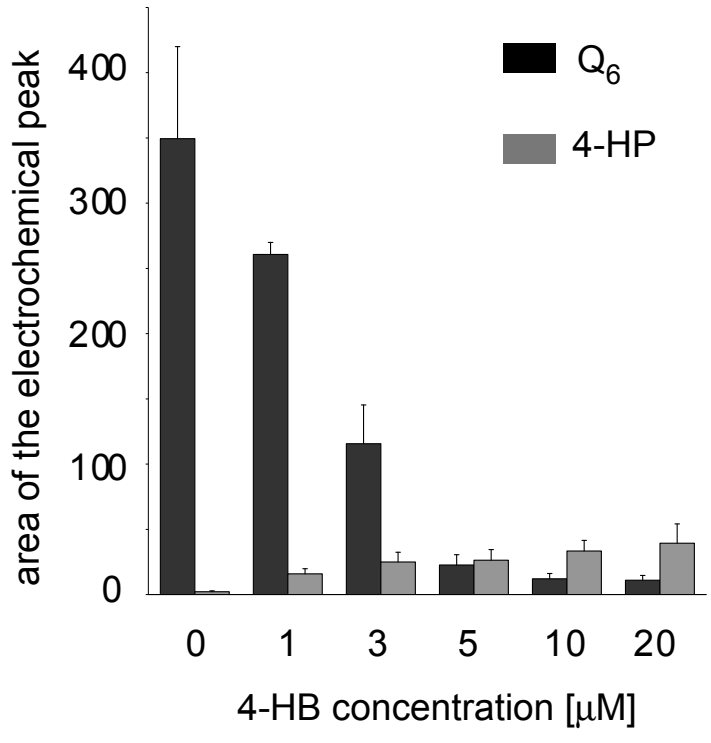

A
4-AP
4-HP
OH
NH2
OH
OH
6
6
Q6
DMQ6
Q4
WT pABA
Δcoq6/Coq8 4-HB
Δcoq6/Coq8 pABA
Δcoq6/vec pABA
electrode response (V)
0
200
400
600
800
1000
elution time (s)
B
Q6
4-HP
4-AP
Q4
DMQ6
Δcoq6
Coq6 pABA
G386A N388D 4-HB
G386A N388D pABA
vec pABA
electrode response (V)
0
200
400
600
800
1000
elution time (s)
C
Q6
Q4
4-AP
4-HP
DMQ6
Δflx1/ Fad1 4-HB
WT pABA
Δflx1 pABA
Δflx1 4-HB
Δflx1
electrode response (V)
0
200
400
600
800
1000
elution time (s)

Figure

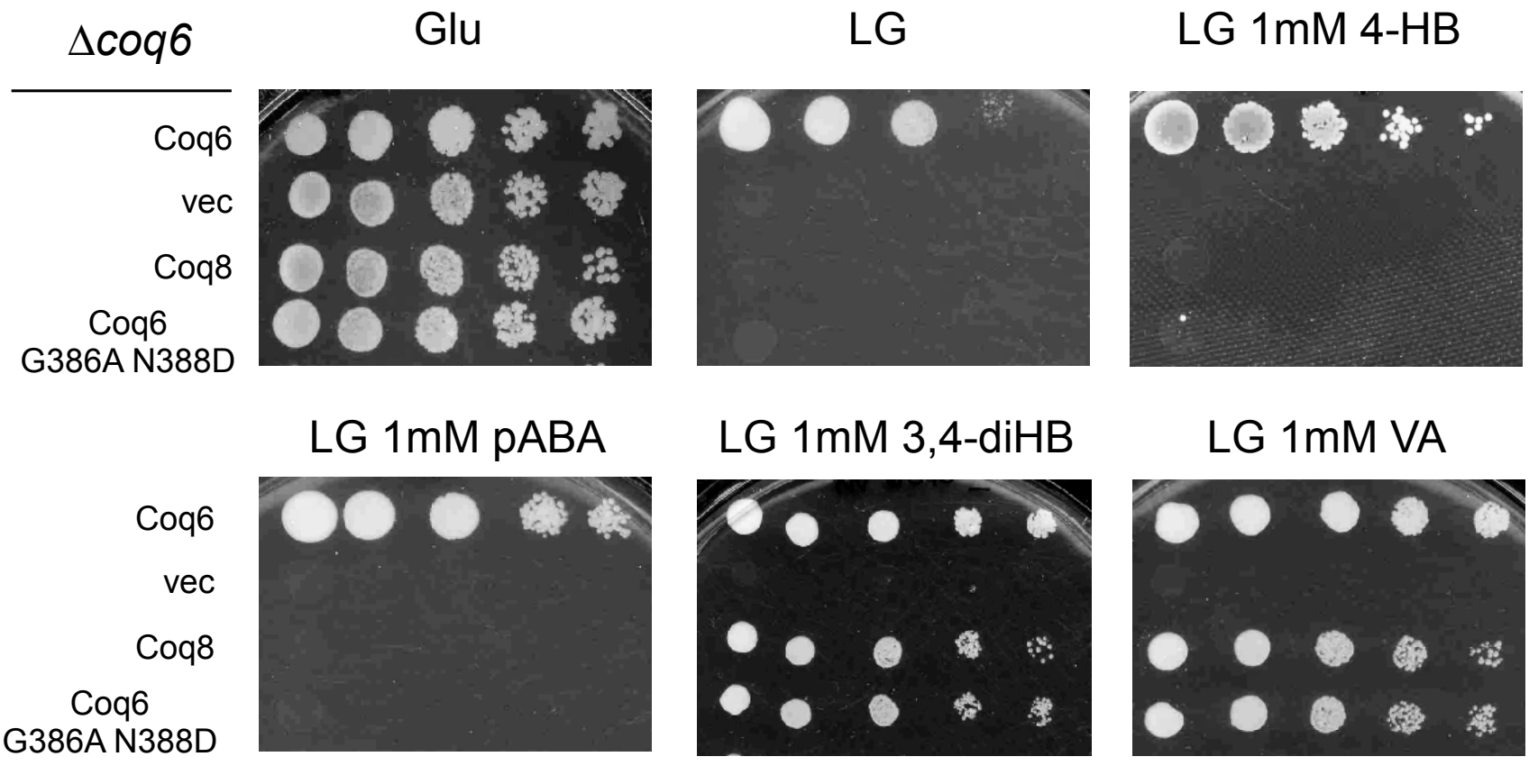
A


DMQ6
Q6
4-AP
4-HP
Q4
electrode response (V)
0
200
400
600
800
1000
elution time (s)
Δcoq6/Coq8
0.5 mM VA
0.1 mM VA
0.1 mM pABA
0.1 mM 4-HB

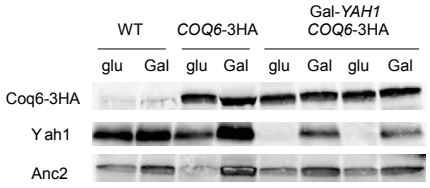
C

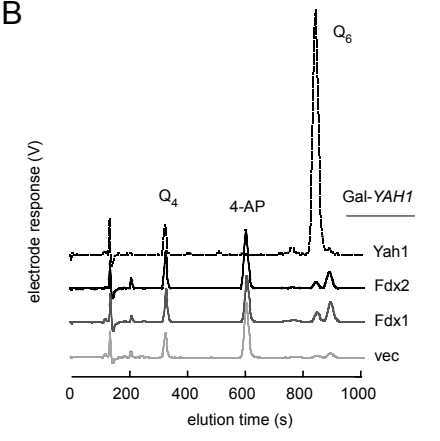

area of the electrochemical peak
400
300
200
100
0
Q6
4-HP
0
1
3
5
10
20
4-HB concentration [μM]

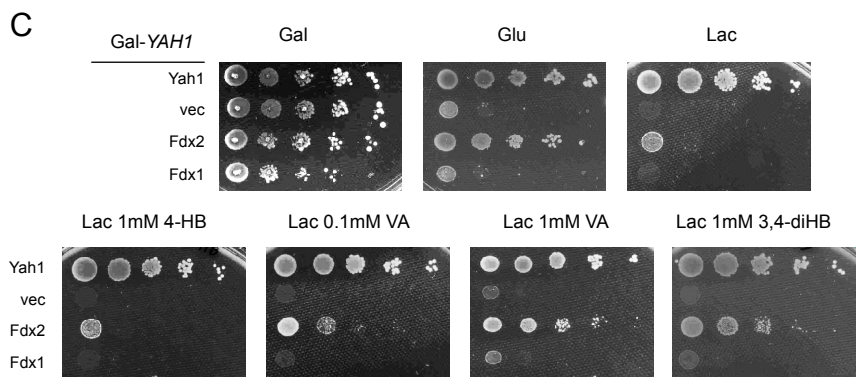
B


Δcoq6
Glu
LG
LG 1mM 4-HB
Coq6
vec
Coq8
Coq6
G386A N388D
LG 1mM pABA
LG 1mM 3,4-diHB
LG 1mM VA
Coq6
vec
Coq8
Coq6
G386A N388D

Figure


WT
COQ6-3HA
Gal-YAH1
COQ6-3HA
glu
Gal
glu
Gal
glu
Gal
glu
Gal
Coq6-3HA
Yah1
Anc2


B
Q6
Q4
4-AP
Gal-YAH1
Yah1
Fdx2
Fdx1
vec
electrode response (V)
0
200
400
600
800
1000
elution time (s)


C
Gal-YAH1
Gal
Glu
Lac
Yah1
vec
Fdx2
Fdx1
Lac 1mM 4-HB
Lac 0.1mM VA
Lac 1mM VA
Lac 1mM 3,4-diHB

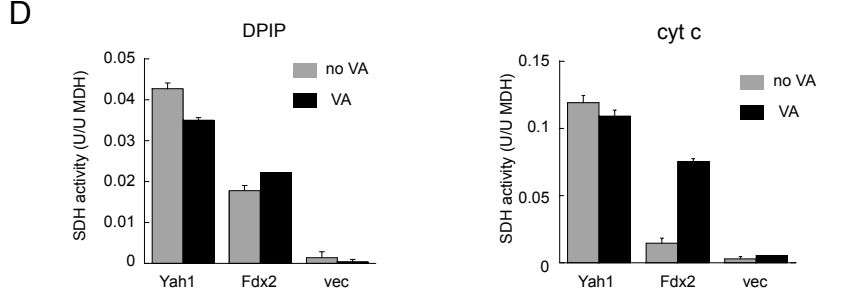
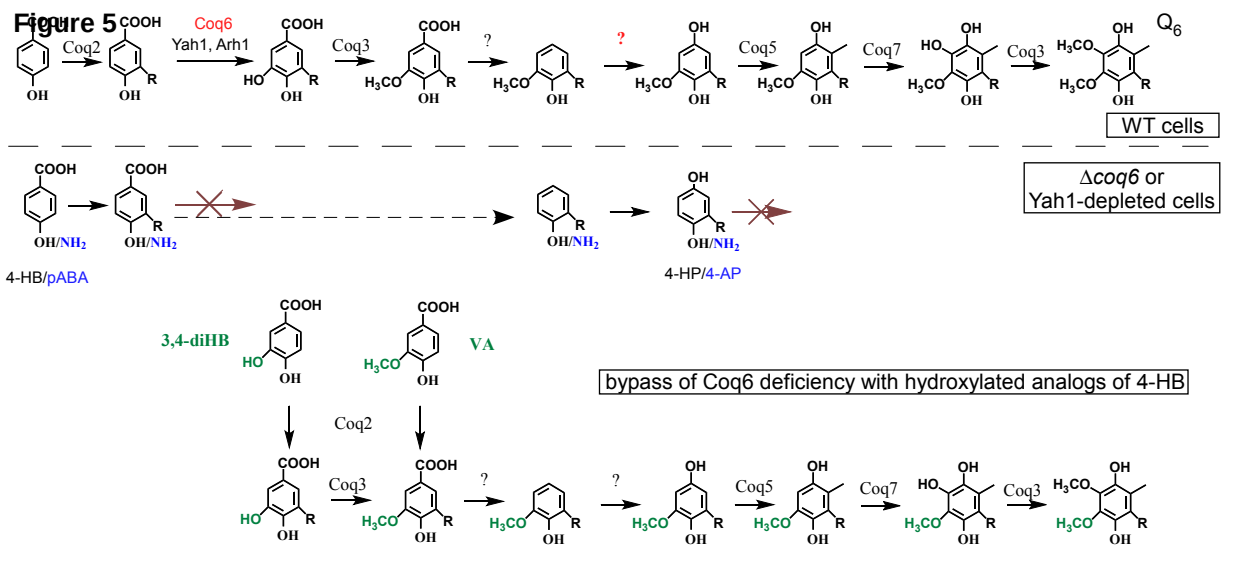

D
DPIP
cyt c
no VA
VA
SDH activity (U/U MDH)
0.05
0.04
0.03
0.02
0.01
0
0.15
0.1
0.05
Yah1
Fdx2
vec

Figure 5


Coq1
Coq2
Coq6
Yah1, Arh1
Coq3
?
?
Coq5
Coq7
Coq3
Q6
WT cells
Δcoq6 or
Yah1-depleted cells
4-HB/pABA
4-HP/4-AP
3,4-diHB
VA
bypass of Coq6 deficiency with hydroxylated analogs of 4-HB
Coq2
Coq3
?
?
Coq5
Coq7
Coq3

Inventory of Supplemental Information

Supplemental information includes four figures and one table.

Figure S1 relates to the growth media used in Figure 2.

Figure S2 is a sequence alignment of Coq6 with other flavine-dependent monooxygenases. It illustrates the residues that we mutated in Coq6. These Coq6 mutants are used in Figure 2.

According to the instructions, Figure S1 and S2 should be grouped together because they both relate to Figure 2, however since the format of Fig. S1 and S2 is different, I took the liberty not to group the figures.

Figure S3 displays the chemical structure of compounds used in Figure 3 and additional experiments complementary to those of Figure 3.

Figure S4 shows data obtained with the Gal-*YAH1* strain which are complementary to those presented in Figure 4.

Table S1 contains the yeast strains used in this study.

Supplemental data

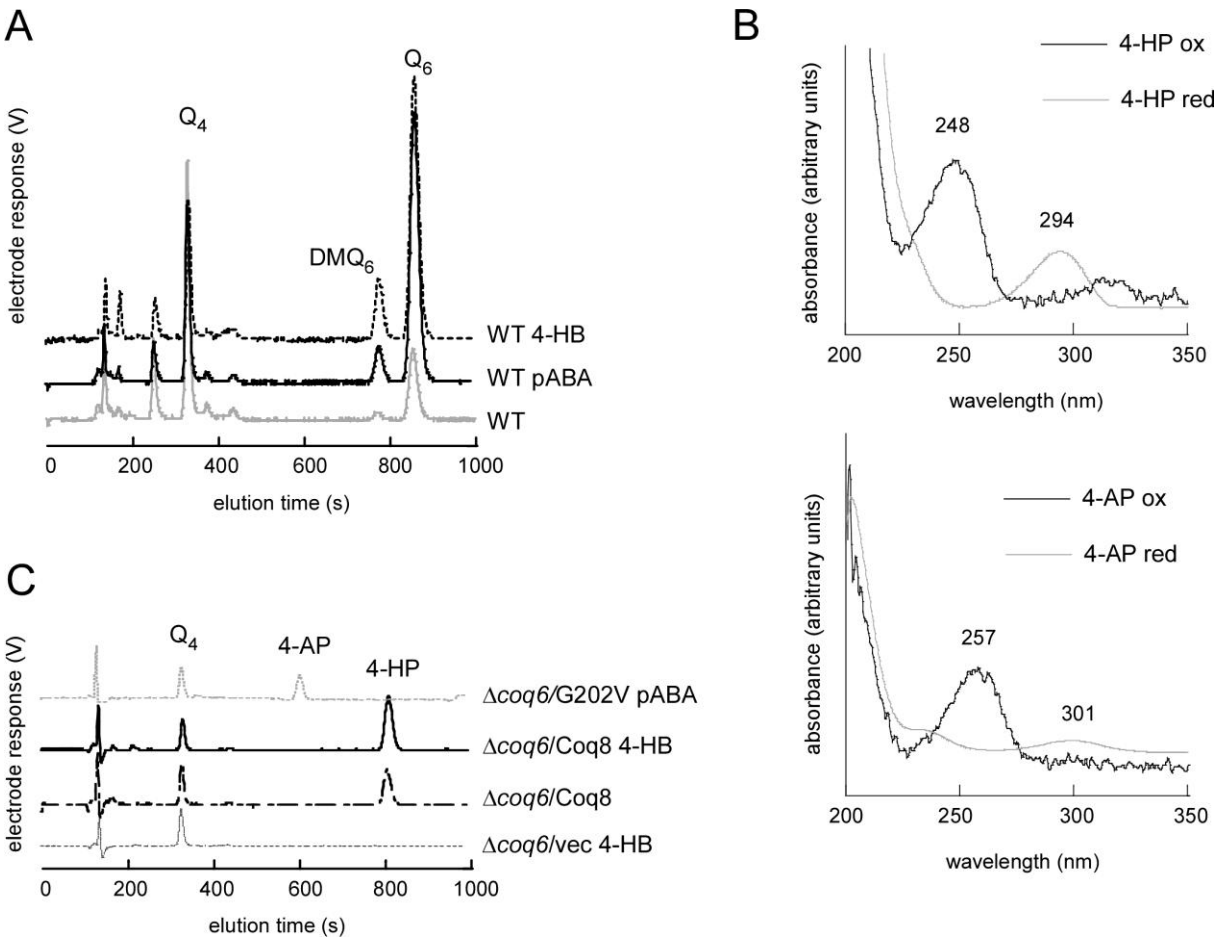


Figure S1


Coq6 MFFSKVMLTRRILVRGLATAKSSAPKLTDVLIVGGGPAGLTLAASIKNSPQLKDLKTTLV 60
UbiH ----------MSVIIVGGGMAGATLALAISR------LSHGAL 27
PobA ----------MKTQVAIIGAGPSGLLLGQLLHK---------AGI 26
Kmo ----------MDSSVIQRKKVAVIGGGLVGSLQACFLAKR-------NFQI 34
Coq6 DMVDLKDKLSDFYNSPPDYFTNRIVSVTPRSIHFLENNAGATLMHDRIQSYDG-LYVTDG 119
UbiH PVHLIEATAPESHAHPG--FDGRAIALAAGTCQQLARIGVWQSLADCATAITT-VHVSDR 84
PobA DNVILERQTPDYVLGRI-----RAGVLEQGMVDLLREAGVDRRMARDGLVHEG-VEIAFA 80
Kmo DVYEAREDTRVATFTRG---RSINLALSHRGRQALKAVGLEDQIVSQGIPMRARMIHSLS 91
Coq6 CSKATLDLARDS-MLCMIEIINIQASLYNRISQYDSKKDSIDIIDNTKVVNIKHSDPNDP 178
UbiH GHAGFVTLAAEDYQLAALGQVVELHNVGQRLFALLRKAPGVTLHCPDRVANVAR-----T 139
PobA GQRRRIDLKRLS--GGKTVTVYGQTEVTRDLMEAREACGATTVYQAAEVRLHDLQ----- 133
Kmo GKKSAIPYGTKS----QYILSVSRENLNKDLLTAAEKYPNVKMHFNHRLLKCNPE----- 142
Coq6 LSWPLVTLS-NGE--VYKTRLLVGADGFNSPTRRFSQIPSRGWMYNAYGVVASMKLEYPP 235
UbiH QSHVEVTLE-SGE--TLTGRVLVAADGTHSALATACGVDWQQEPYEQLAVIANVATS-VA 195
PobA GERPYVTFERDGERLRLDCDYIAGCDGFHG-ISRQSIPAERLKVFERVYPFGWLGLLADT 192
Kmo -EGMITVLGSDKVPKDVTCDLIVGCDGAYSTVRSHLMKKPRFDYSQQYIPHGYMELTIPP 201
Coq6 FKLR-----GWQRFLPTGPIAHLPMPENNATLVWSSSERLSRLLLSLPPESFTALINAAF 290
UbiH HEGR-----AFERFTQHGPLAMLPMSDGRCSLVWCHPLERREEVLSWSDEKFCRELQSAF 250
PobA PPVS-----HELIYANHPRGFALCSQRSATRSRYYVQVPLSEKVEDWSDERFWTELKARL 247
Kmo KNGDYAMEPNYLHIWPRNTFMMIALPNMNKSFTCTLFMPFEEFEKLLTSNDVVDFFQKYF 261
Coq6 VLEDADMNYYYRTLEDGSMDTDKLIEDIKFRTEEIYATLKDESDIDEIYPPRVVSIIDKT 350
UbiH GWRLGKITHAGK---------- 262
PobA PSEVAEKLVTGPS---------- 262
Kmo PDAIPLIGEKLLVQD---------- 280
Coq6 RARFPLKLTHADRYCTDRVALVGDAAHTTHPLAGQGLNMGQTDVHGLVYALEKAM----- 405
UbiH RSAYPLALTHAARSITHRTVLVGNAAQTLHPIAGQGFNLGMRDVMSLAETLTQAQ----- 317
PobA KSIAPLRSFVVEPMQHGRLFLAGDAAHIVPPTGAKGLNLAASDVSTLYRLLLKAY----- 317
Kmo PAQPMISVKCSSFHFKSHCVLLGDAAHAIVPFFGQGMNAGFEDCLVFDELMDKFSNDLSL 340
Coq6 ----------ERGLDIGSSLSLEPFWAERYPSN----------NVL 431
UbiH ----------ERGEDMGDYGVLCRYQQRRQSDR----------EAT 343
PobA ----------REGRGE-LLERYSAICLRRIWKA----------ERF 342
Kmo CLPVFSRLRIPDDHAISDLSMYNYIEMRAHVNSSWFIFQKNMERFLHAIMPSTFIPLYTM 400
Coq6 LGMADKLFKLYHTNFPPVVALRTFGLNLTNKIGPVKNMIIDTLGGNEK---------- 479
UbiH IGVTDSLVHLFANRWAPLVVGRNIGLMTMELFTPARDVVAQRTLGWVAR---------- 392
PobA SWWMTSVLHRFPDTDAFSQRIQQTELEYYLGSEAGLATIAENYVGLPYEEIE-------- 394
Kmo VTFSRIRYHEAVQRWHWQKKVINKGLFFLGSLIAISSTYLLIHYMSPRSFLRLRRPWNWI 460
Coq6 ----------
UbiH ----------
PobA ----------
Kmo AHFRNTTCFPAKAVDSLEQISNLISR 486

Figure S2

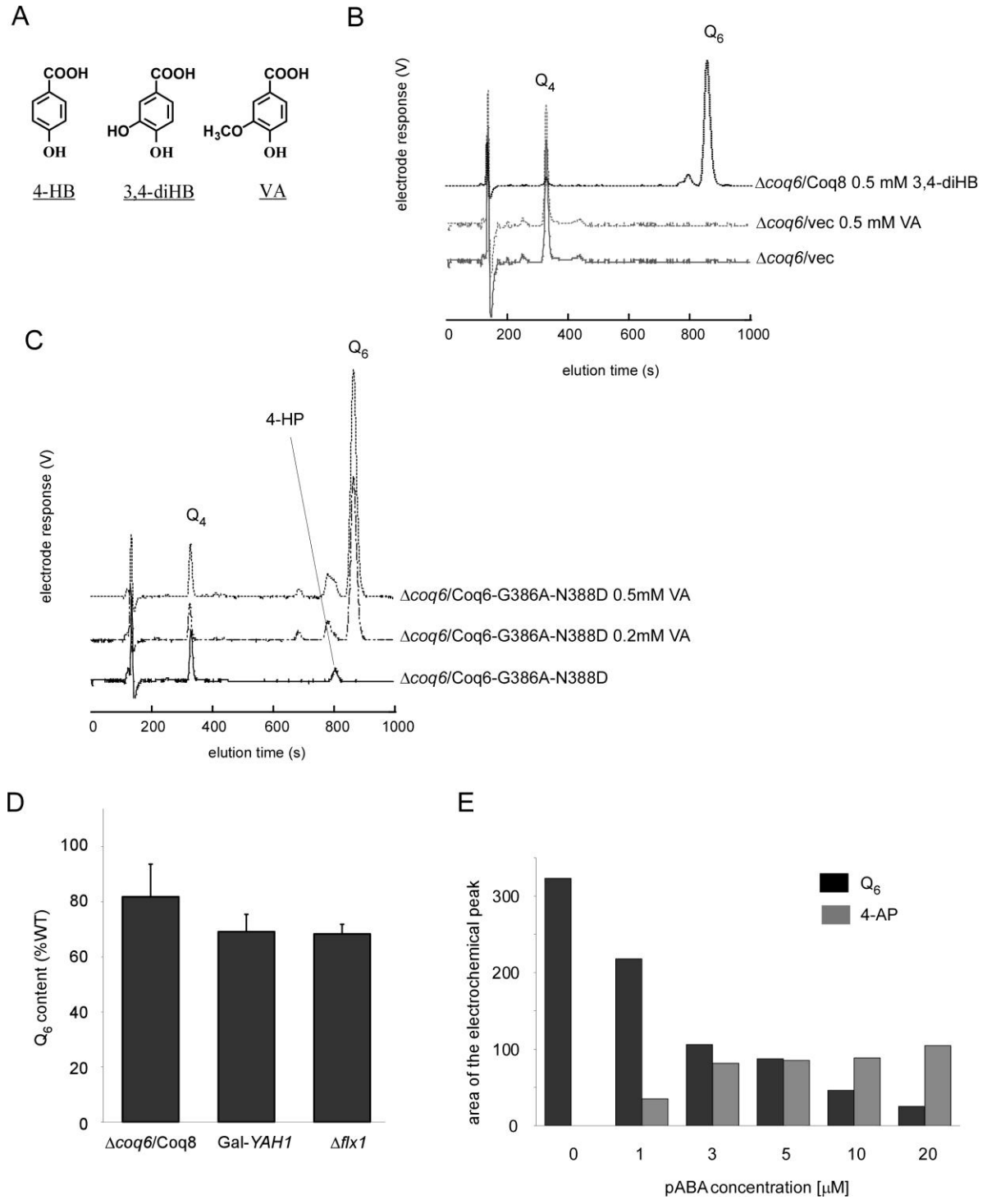

A
COOH
OH
4-HB
COOH
HO
OH
3,4-diHB
COOH
H3CO
OH
VA
B
Q6
Q4
electrode response (V)
Δcoq6/Coq8 0.5 mM 3,4-diHB
Δcoq6/vec 0.5 mM VA
Δcoq6/vec
0
200
400
600
800
1000
elution time (s)
C
Q6
4-HP
Q4
electrode response (V)
Δcoq6/Coq6-G386A-N388D 0.5mM VA
Δcoq6/Coq6-G386A-N388D 0.2mM VA
Δcoq6/Coq6-G386A-N388D
0
200
400
600
800
1000
elution time (s)
D
Q6 content (%WT)
100
80
60
40
20
0
Δcoq6/Coq8
Gal-YAH1
Δflx1
E
area of the electrochemical peak
300
200
100
0
Q6
4-AP
0
1
3
5
10
20
pABA concentration [μM]

Figure S3

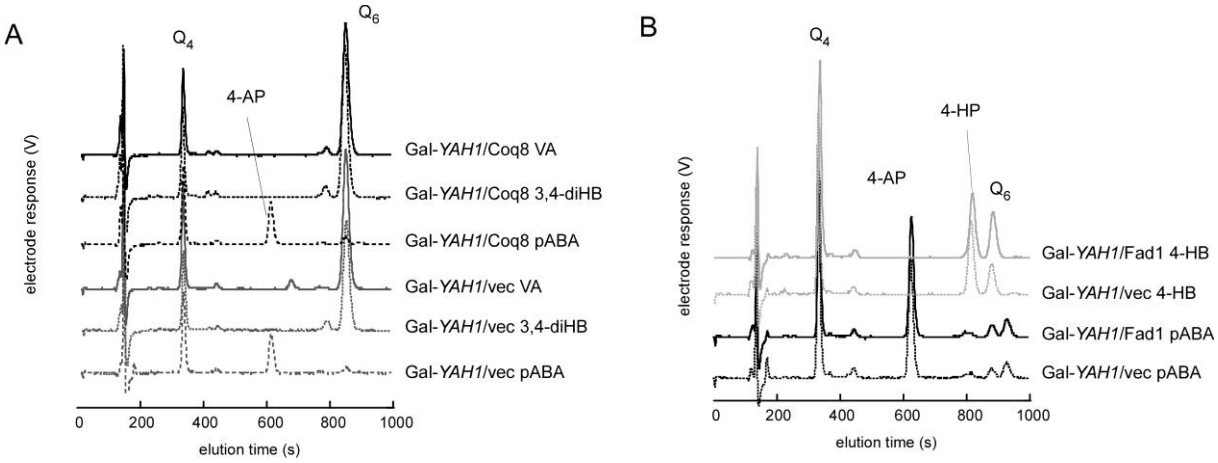

A
Q4
Q6
4-AP
electrode response (V)
Gal-YAH1/Coq8 VA
Gal-YAH1/Coq8 3,4-diHB
Gal-YAH1/Coq8 pABA
Gal-YAH1/vec VA
Gal-YAH1/vec 3,4-diHB
Gal-YAH1/vec pABA
0
200
400
600
800
1000
elution time (s)
B
Q4
4-HP
4-AP
Q6
electrode response (V)
Gal-YAH1/Fad1 4-HB
Gal-YAH1/vec 4-HB
Gal-YAH1/Fad1 pABA
Gal-YAH1/vec pABA
0
200
400
600
800
1000
elution time (s)

Figure S4

Strain	Genotype	Source
BY4741	MAT a, *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	euroscarf
Gal-*YAH1*	*ade2-1 his3-1,15 leu2-3,112 trp1-1, ura3-1,* Gal-*YAH1::LEU2*	(Lange et al., 2000)
Δ*coq6*	MATα; *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 COQ6::kanMX4*	euroscarf
COQ6-3HA	*his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 COQ6-3HA::HIS3*	This work
COQ6-3HA Gal-*YAH1*	*his3 ura3 COQ6*-3HA::*TRP1,* Gal-*YAH1::LEU2*	This work
Δ*flx1*	MAT a, *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 FLX1::kanMX4*	euroscarf

Table S1 : Yeast strains used in this study

Figure S1 related to Figure 2: A) WT cells were grown for 24 hours to stationary phase in glucose synthetic medium without pABA supplemented or not with 0.1 mM pABA or 0.1 mM 4-HB as indicated. Extracts of 2 mg of cells (no addition) or 1.5 mg of cells (4-HB and pABA) were analyzed by HPLC-ECD. B) Purified 3-hexaprenyl-4-aminophenol (4-AP) or 3-hexaprenyl-4-hydroxyphenol (4-HP) were injected on the HPLC column with the precolumn electrode set at +550mV (ox) or -500mV (red). The UV-vis spectra of the oxidized (ox) and reduced (red) forms of 4-AP and 4-HP were recorded at the apex of the elution peak. Absorption maxima of the spectra are indicated. C) Δ*coq6* cells transformed with an episomal vector coding for Coq8, with an empty vector (vec) or with a centromeric vector coding for Coq6-G202V were grown in glucose synthetic medium without pABA containing or not 100 µM pABA or 100 µM 4-HB. Lipid extracts of 4 mg of cells were analyzed by HPLC-ECD.

Figure S2: Sequence alignment of different Class A flavoprotein monooxygenases. Alignments were generated with ClustalW2. The proteins are the Coq6 monooxygenase from *Saccharomyces cerevisiae* (AAB61341), the 2-polyprenyl-6-methoxyphenol 4-hydroxylase from *Escherichia coli* (UbiH, BAA14326), the P-hydroxybenzoate hydroxylase from

Pseudomonas aeruginosa (PobA, P20586) and the kynurenine 3-monooxygenase from *Homo sapiens* (Kmo, NP_003670). The conserved residues that we mutated in Coq6 (G202, G386 and N388) are highlighted. The DG motif (D201, G202 in Coq6) has been proposed to bind the pyrophosphate moieties of both FAD and NADPH in PobA (Eppink et al., 1997) whereas the N300 from PobA is in contact with the isoalloxazine ring of the active site FAD (Palfey et al., 1994).

Figure S3 related to Figure 3: A) Chemical structure of 4-HB, 3,4-dihydroxybenzoic acid (3,4-diHB) and vanillic acid (VA). B) Δ*coq6* cells transformed with an empty vector (vec) or an episomal vector coding for Coq8 were grown in glucose synthetic medium without pABA containing or 0.5 mM vanillic acid (VA) or 0.5 mM 3,4-dihydroxybenzoic acid (3,4-diHB). Lipid extracts of 8 mg of cells (vec) or 4 mg of cells (Coq8) were analyzed by HPLC-ECD. C) Δ*coq6* cells transformed with a centromeric vector coding for Coq6-G386A-N388D were grown in glucose synthetic medium without pABA containing or not the indicated concentration of VA. Lipid extracts of 3 mg of cells were analyzed by HPLC-ECD. D) Q_6 content of Δ*coq6* strain overexpressing Coq8, of Gal-*YAH1* and Δ*flx1* strains grown in glucose synthetic medium without pABA containing 1 mM VA compared to WT cells grown in the same medium. Q_6 was quantified by integrating the peak of Q_6 on electrochromatograms and correction for eventual loss during the extraction procedure was made based on the peak of the Q_4 standard. The error bars represent standard deviation (n=3). E) Quantification of Q_6 and 3-hexaprenyl-4-aminophenol (4-AP) formed in Δ*coq6* cells containing an episomal vector coding for Coq8 grown in glucose synthetic medium without pABA with 1mM VA and the indicated concentration of pABA.

Figure S4 related to Figure 4: A) Gal-*YAH1* cells transformed with an empty vector (vec) or an episomal vector coding for Coq8 were grown for 24 hours in synthetic medium without pABA supplemented with glucose and 0.1 mM pABA or 0.1 mM 3,4-dihydroxybenzoic acid (3,4-diHB) or 0.1 mM vanillic acid (VA). Extracts of 1.5 mg of cells were analyzed by HPLC-ECD. B) Gal-*YAH1* cells transformed with an empty vector (vec) or an episomal vector coding for Fad1 were grown for 24 hours in synthetic medium without pABA supplemented with glucose and 1mM pABA or 1mM 4-HB. Extracts of 4 mg of cells were analyzed by HPLC-ECD.